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Formation of Reducing Substances in Pea Seeds ¹

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INTRODUCTION

Hopkins and Morgan, from the results of isolation procedures, concluded (1) that considerable reduced glutathione (GSH) was produced during the initial stages of germination of dry pea seeds, and that the amount present decreased as germination proceeded. When they incubated pea powder anaerobically with phosphate buffer at pH 6.0 they found a marked increase in reducing power which they assumed to be due to glutathione formation. The changes appeared to be enzymatically induced, and it seemed desirable to learn more of the enzymes involved.

Toward this end, a study has been made of the changes, using more specific methods. The results show that increased reducing power during germination of whole peas and incubation of pea preparations is due largely to formation of substances other than glutathione.

METHODS AND MATERIALS

Reduced glutathione (GSH) was determined using the glyoxalase method of Schroeder and Woodward (2). The method is quite specific, and recovery experiments gave satisfactory results. The GSH levels in peas are such that the determinations were near the lower limit of usefulness of the method, and experiments indicated a precision of $\pm 10\%$.

Reduced ascorbic acid was determined using the method of Hochberg, Melnick, and Oser (3). Recoveries were satisfactory, and GSH, at the levels found present in the samples, did not interfere, although it did in direct titration and xylene-extraction methods.

Free cysteine and cystine were determined by the method of Sullivan, Hess, and Howard (4) with minor modifications suggested by Neubeck and Smythe (5). The

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method is specific. In particular, GSH gives no color. However, some material in pea extracts does tend to suppress the color, as recoveries of added cysteine were only 70-80% complete. The situation was not improved by previous isolation of the cysteine as the cuprous salt (6) or by previous aeration of extracts (7). However, it seems most unlikely that more accurate figures would cause any great alteration in the relative changes or in the conclusions derived from them.

Titration with 0.005 *N* iodine was used as a rough measure of the total reducing power.

The peas used were dried Alaska peas (*Pisum sativum*) of the 1948 crop.

RESULTS

When it was found that extracts prepared from peas increase in reducing power toward iodine during anaerobic incubation, it appeared likely that such preparations would be a convenient and worth-while material for study. The supernatant solution obtained after grinding 10 parts of pea meal with 40 parts of 0.045 *M* phosphate buffer of pH 6 in a mortar, and centrifuging, was allowed to stand at room temperature for 24 hr. under toluene and under anaerobic conditions. Reactions were stopped by adding metaphosphoric acid either at the beginning or at the end of the incubation period and determinations were run on the supernatant solution after centrifuging. It was found that while the iodine titration was approximately doubled in 24 hr. no change occurred in the GSH level. No ascorbic acid could be detected either before or after incubation. The cysteine level, however, became four or five times as high after incubation as before. Little or no cystine was found. Repeated experiments gave similar results, and Table I shows a representative case. In previously boiled extracts, no changes occurred in any of the constituents for which analyses were made. The increase of cysteine is undoubtedly real, in spite of the inaccuracy of the determinations, and the relative increase is believed to be not far wrong. The increase is probably due to the proteolytic activity in peas (6). It is not possible to tell whether the increase accounts for all the increase in iodine titration, because of the uncertainty of the absolute cysteine level, the unsatisfactory nature of the end point in the iodine titration, and the fact that the iodine equivalent of cysteine varies with many factors (7).

These results with extracts raised a question as to the interpretation of the experiments of Hopkins and Morgan (1) with pea suspensions. Experiments were made by incubating at room temperature for 24 hr. under toluene and anaerobic conditions 3.75 g. of ground peas in 15 ml. of 0.045 *M* phosphate buffer of pH 6. Here again the iodine titration

approximately doubled, and cysteine increased four to six times, while no cystine appeared. Glutathione not only did not increase, but actually decreased to less than half the original level. Again no ascorbic acid developed. Figures for a representative experiment are given in Table I.

In order to determine whether corresponding changes occurred during the germination of peas, 10-g. lots were allowed to germinate for selected periods of time between sheets of moistened absorbent paper in a cabinet in a humid atmosphere at 20°C. When removed, the peas were weighed, thoroughly ground in a mortar with 60 ml. of 3% metaphosphoric acid, and centrifuged. The residue was washed twice with 20-ml. portions of acid, and the washings were combined with the first supernatant solution. Experiments showed this procedure to give substantially complete extraction of the GSH, and, presumably, of the other soluble constituents. The results of similar experiments in this case were much more variable than in the experiments described above, since the variability between different lots of peas is involved, as well as the precision of the methods.

TABLE I
Changes on Incubation of Pea Preparations

| Experimental material | GSH, $\mu\text{g.}/\text{g. peas}^a$ | | Cysteine, $\mu\text{g.}/\text{g. peas}$ | | Ml. 0.005 <i>N</i> iodine reduced/g. peas | |
|-----------------------|--------------------------------------|-------|---|-------|---|-------|
| | Initial | Final | Initial | Final | Initial | Final |
| Pea extract | 210 | 205 | 15 | 75 | 0.43 | 0.82 |
| Pea suspension | 190 | 90 | 25 | 105 | 0.37 | 0.80 |

^a Complete extractions of soluble constituents were not attempted.

^b Cysteine recoveries were only 70–80%, so these figures are probably too low.

Cysteine did not increase in germinating peas. A small amount was present in all samples—too small for precise quantitative analysis. In nearly every series, however, the cystine level would begin to show a marked rise at from 4 to 8 days' germination. Figures for changes in GSH and ascorbic acid are presented in Table II. Statistical considerations indicate the only probably significant differences in GSH contents are those between the initial level and the 2- and 4-day levels. The development of about the same level of ascorbic acid has been noted before (8,9,10), and is more than sufficient to account for the small and highly variable rise and subsequent fall in iodine titration.

TABLE II
Changes during Germination of Peas

Germination between sheets of moist paper at 20°C.; analytical results based on weight of dry peas used.

| Time of germination | Initial | 24 hr. | 2 days | 4 days | 6 days | 8 days |
|--|--------------|--------------|--------------|--------------|--------------|--------|
| Glutathione, ^a $\mu\text{g.}/\text{g.}$ | 540 \pm 53 | 590 \pm 42 | 660 \pm 98 | 690 \pm 51 | 610 \pm 98 | 450 |
| No. of GSH determinations | 5 | 4 | 5 | 6 | 5 | 2 |
| Ascorbic acid, $\mu\text{g.}/\text{g.}$ | 0 | 5, 10 | 60 | 325, 335 | 155 | |
| Cystine, ^b $\mu\text{g.}/\text{g.}$ | present | present | 0-40 | 0-85 | 0-135 | 55-190 |

^a Mean and standard deviation.

^b The method gave recoveries of only 70-80%, so these figures are probably too low.

The possibility that some of the changes during germination might be due to bacterial action was considered. Two series were germinated at 20°C. in Petri dishes on sterile agar after surface disinfection of the peas with sodium hypochlorite (1% available chlorine), followed by two rinsings in sterile water. Under these conditions germination was much slower, as indicated by increasing wet-weight, and the changes were delayed. However the changes were about the same as in the other germination experiments; namely, a small increase in GSH, no apparent change in cysteine or cystine in the 8-day period, and a large increase in ascorbic acid (in fact to about twice the maximum previously found).

CONCLUSIONS

The belief that glutathione is synthesized rapidly in germinating peas, and later largely disappears would appear to be erroneous. The concentration changed little throughout the period under study here. Changes in reducing power toward iodine were not an indication of changes of GSH level in any of the materials used in this work.

Indications are that proteolytic activity is held in check during early germination, though of course its effect may be counterbalanced by other reactions. The mechanisms for ascorbic acid synthesis were no longer active when the cell structure of the pea was destroyed.

SUMMARY

During anaerobic incubation at pH 6 of pea extracts or suspensions of ground peas, the reducing power toward iodine doubled, and the free

cysteine content increased several-fold. No cystine or ascorbic acid developed. The glutathione level remained unchanged in the extracts and, contrary to published reports, more than half the original glutathione disappeared in the suspensions.

During germination of peas in the dark, the increase in reducing power could be accounted for by the formation of ascorbic acid. A small increase of glutathione above the original level of about 540 $\mu\text{g./g.}$ of peas occurred during the early stages of germination. Cysteine was not formed but free cystine began to accumulate at from 4 to 8 days germination.

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Is Hemicellulose Utilized by the Rat?

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INTRODUCTION

It has been generally presumed that some of the carbohydrate compounds found in vegetable food materials other than sugar and starch are utilized by the body and it has been supposed that the hemicellulose fraction includes such compounds. Green-leaf vegetables are important items in the dietary in China. Chinese celery cabbage, for example, contains negligible amounts of starch, but Ting and Adolph (1), using phlorhizinized rats, have estimated that 38% of its total carbohydrate content is available to the rat. It is possible that, in areas where the food intake is essentially vegetarian, utilizable carbohydrate from leaf vegetables constitutes a not inconsiderable portion of the total daily caloric intake. We have made two preparations of hemicellulose, one from wood, another from cabbage, and have estimated utilization by measuring the amount of liver glycogen formed on feeding this material to rats.

EXPERIMENTAL

In preparing the hemicellulose, the method of Anderson and co-workers (2) was followed. For Prepn. 1, hardwood sawdust, mainly oak and maple, was used as the source of hemicellulose. The material, in 1-kg. lots was extracted with hot water and then treated twice with 3 l. of 4% sodium hydroxide. In each case the material was let stand overnight, filtered through cloth, and the filtrate slightly acidified with concentrated hydrochloric acid and treated with 1.5 volumes of 95% alcohol. The precipitated hemicellulose was redissolved in 4% sodium hydroxide, centrifuging off the residue, the solution being neutralized with hydrochloric acid, purified by treatment with bromine, and pre-

cipitated by the addition of alcohol. The precipitate was then washed by repeated treatment with 85% alcohol, with centrifuging, till free from chloride, the final treatment being with 95% alcohol followed by drying in a desiccator. From 3.2 kg. of sawdust, a total of 28.4 g. of hemicellulose was prepared. For Prepn. 2, desiccated cabbage (*Brassica oleracea capitata*) was used as the raw material and a procedure similar to the above was followed. From 2.8 kg. of the desiccated cabbage, a total of 13.4 g. of the dried hemicellulose preparation was obtained. Both preparations were starch-free and of a grayish-white color.

TABLE I
Liver Glycogen in Rats Fed Hemicellulose

| Carbohydrate fed | Number of animals | Glycogen content of liver | | Standard deviation |
|-----------------------------|-------------------|---------------------------|---------|--------------------|
| | | Range | Average | |
| Series 1 | | % | % | |
| Starch | 11 | 0.37-1.81 | .92 | .37 |
| Hemicellulose (Prepn. 1) | 11 | 0.19-0.55 | .29 | .13 |
| None | 11 | 0.09-0.44 | .21 | .10 |
| Series 2 | | | | |
| Starch | 11 | 0.43-1.82 | 1.03 | .42 |
| Hemicellulose (Prepn. 2) | 11 | 0.10-0.39 | .22 | .10 |
| None | 11 | 0.14-0.37 | .23 | .08 |

In measuring the conversion of the hemicellulose into glycogen by the rat, the usual technique was employed. Litter-mate adult rats, males, weighing 160-190 g. were divided as evenly as possible into three groups and starved for 48 hr. Groups 1 and 2 were then fed starch and hemicellulose, respectively, 200 mg./100 g. body weight, mixed in each case with 80 mg. of cocoa butter; group 3 was fed the cocoa butter only. Any rat which did not eat the portion within 15 min. was discarded. Exactly 4 hr. after the test material was administered, the rats were killed by a blow on the head, the liver promptly removed, and the glycogen content determined by the Somogyi method. At the same time, the stomach was opened and examined, and if the stomach still contained undigested material, this animal was discarded. The measure-

ments with Prepn. 1 were carried out in Ithaca, those with Prepn. 2 in Peking. The results are shown in Table I.

Whereas the starch-fed animals showed the usual appreciable amounts of glycogen deposited in the liver, there was no significant difference between the rats fed hemicellulose and those fed no carbohydrate at all. It is evident that hemicellulose prepared as indicated cannot be regarded as a nutrient for the rat.

SUMMARY

Laboratory preparations of hemicellulose from hardwood and from cabbage do not serve as a source of glycogen in the rat.

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The Locus of Action of α -Tocopheryl Phosphate in the Succinoxidase System

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INTRODUCTION

The oxygen consumption of muscle from vitamin-E-deficient animals is considerably greater than that of normal muscle (1-6) and is quickly restored to the normal level if α -tocopherol is given orally or if the water-soluble α -tocopheryl phosphate (α -TPh) is injected (4,5). Of considerable interest, therefore, is a study of the action of α -TPh on enzyme systems of muscle. Houchin (7) was the first to report an inhibition of the succinoxidase system by α -TPh. Ames (8), studying the mechanism of this inhibition, concluded that α -TPh exerts its effect by precipitating calcium, thus removing an important ingredient of the succinoxidase system. According to his view, the removal of calcium ions as the insoluble calcium salt of α -TPh deprives diphosphopyridine nucleotidase of its activator. As a result, cozymase, the coenzyme for malic dehydrogenase, is preserved and oxidation of malate to oxalacetate takes place. Oxalacetate, however, is a potent inhibitor of succinic dehydrogenase, so that suppression of the succinoxidase system is the net result of this chain of interlocking events.

Since α -TPh is precipitated by calcium in the succinoxidase system, any direct effect which the α -TPh may have on the enzymes in this system is minimized. Therefore, it would be desirable to study the effect of α -TPh on the succinoxidase system without added calcium in order to ascertain if a direct effect can be demonstrated. It is possible to obtain nearly maximal succinoxidase activity without the addition of calcium by simply allowing a tissue homogenate to stand for several hours in the cold (9,10). The "spontaneous activation" which occurs is

supposed to result from the enzymatic degradation of cozymase which proceeds slowly even in the absence of calcium.

This paper deals with the effect of α -TPh on the "spontaneously activated" succinoxidase system to which no calcium has been added.

EXPERIMENTAL

Liver or muscle in these experiments was obtained from adult white rats of either sex, which were stunned by a blow on the head and decapitated. The organ was rapidly excised, rinsed in ice-cold glass-redistilled water, and homogenized immediately in water by the Potter-Elvehjem procedure (11). Manometric determinations were made with a Warburg constant volume respirometer at 38°C. The center wells of the Warburg flasks contained 0.2 ml. of 10% KOH and a small filter-paper roll. The gas phase was air. Manometric readings were recorded at 10-min. intervals for 1 hr. All solutions employed were prepared in glass-redistilled water. Varying amounts of a solution of α -TPh, adjusted to pH 7.4, were added to each experimental flask to secure the final desired concentration. Two preparations of α -TPh were used, one synthesized in this laboratory and another kindly supplied by Hoffmann-La Roche, Inc.

Succinoxidase activity was measured by the method of Schneider and Potter (12) except that the CaCl_2 and AlCl_3 were omitted. Nearly maximal succinoxidase activity was obtained by allowing the homogenate to remain at 5°C. for 4-6 hr. before use (9,10).

The components of the final reaction mixture in each Warburg flask were as follows: 1 ml. of 0.1 *M* phosphate buffer, pH 7.4; 0.3 ml. of 0.5 *M* sodium succinate, pH 7.4; 0.2 ml. of 2×10^{-4} *M* cytochrome c; 0.1 ml. of 10% liver homogenate or 0.2 ml. of 10% muscle homogenate; and water to make 3 ml. Cytochrome c was prepared from beef hearts by the method of Keilin and Hartree (13) and dialyzed against glass-redistilled water.

Succinic dehydrogenase activity was measured by replacing the cytochrome c in the above system with 0.1 ml. of 0.5% methylene blue.

Cytochrome c and cytochrome oxidase activities were measured by the method of Schneider and Potter (12) for cytochrome oxidase, employing a low and a high concentration of cytochrome c, respectively. Employing the cytochrome oxidase system with a low concentration of cytochrome c (equal to that used in the succinoxidase assay), cytochrome c becomes the limiting factor in the system and the oxygen uptake is determined by the activity of the cytochrome c. On the other hand, in the procedure of Schneider and Potter, as usually employed with a high concentration of cytochrome c, cytochrome oxidase is the limiting factor determining the oxygen uptake in the system. Accordingly, each Warburg flask contained 1 ml. of 0.1 *M* phosphate buffer, pH 7.4; 0.3 ml. of 0.114 *M* ascorbate, pH 7.4; either 0.2 ml. (low) or

1.2 ml. (high) of $2 \times 10^{-4} M$ cytochrome c; 0.2 ml. of 1% liver homogenate; and water to make 3 ml. Autoxidation of the ascorbate was measured by omitting cytochrome c from the reaction mixture. Schneider and Potter report that the oxygen uptake in the absence of cytochrome c is about the same as the rate of autoxidation. Aluminum chloride, which Schneider and Potter include in the final reaction mixture, was not added because it would combine with α -TPh.

RESULTS

The succinoxidase system in the absence of calcium and aluminum is very strongly inhibited by α -TPh. In the case of liver, a concentration of $1 \times 10^{-3} M$ α -TPh produces virtually complete suppression, while even a concentration of $1 \times 10^{-4} M$ causes 83–98% inhibition. When the inhibition at this concentration is calculated for each 10-min. interval, it is evident that frequently the inhibition progressively increases with time and practically reaches a maximum in about 0.5 hr. Even $5 \times 10^{-5} M$ α -TPh exerts a 10–56% inhibition. Similar results were obtained with muscle homogenates. Typical results are illustrated in Table I. It is of interest that very low concentrations of α -TPh ($2.5 \times 10^{-5} M$ to $5 \times 10^{-6} M$) actually stimulate the succinoxidase system (Table II). It may also be noted that the inhibition produced by $1 \times 10^{-4} M$ α -TPh could not be reversed by $1 \times 10^{-3} M$ CaCl_2 when the calcium chloride was tipped in from the side arm 20 min. after the start of the experiment (Table III). If, however, at the start

TABLE I

The Effect of α -Tocopheryl Phosphate on the Succinoxidase System^a

| Time | Liver homogenate | | | | Muscle homogenate | | | |
|---------------------|------------------|----------------------|----------------------|----------------------|-------------------|----------------------|----------------------|----------------------|
| | Control | $5 \times 10^{-5} M$ | $1 \times 10^{-4} M$ | $1 \times 10^{-3} M$ | Control | $5 \times 10^{-5} M$ | $1 \times 10^{-4} M$ | $1 \times 10^{-3} M$ |
| <i>min.</i> | | | | | | | | |
| 20 | 27.8 | 26.2 | 4.7 | 1.5 | 10.5 | 6.8 | 2.0 | 1.0 |
| 40 | 55.6 | 40.2 | 8.5 | 2.2 | 20.2 | 9.7 | 2.8 | 1.4 |
| 60 | 85.0 | 47.9 | 9.6 | 2.6 | 29.1 | 11.2 | 3.0 | 1.7 |
| Per cent inhibition | | 43.7 | 88.7 | 97.0 | | 61.5 | 82.8 | 94.2 |

^a Values in this and succeeding tables represent cubic millimeters of O_2 consumed/mg. dry weight of tissue.

of the experiment, CaCl_2 was added just before the homogenate, which was the last to be added, even $4 \times 10^{-4} M$ CaCl_2 usually caused considerable reversal of inhibition (Table IV). Despite the marked inhibitory effect of α -TPh, it was observed that, if the homogenate itself was made $1 \times 10^{-4} M$ with respect to α -TPh and the mixture allowed to stand for 0.5 hr. at room temperature, no inhibition of the succinoxidase activity of this homogenate occurred.

TABLE II

The Effect of Very Low Concentrations of α -Tocopheryl Phosphate on Liver Succinoxidase

| Time | Control | $5 \times 10^{-6} M$ | $2.5 \times 10^{-5} M$ | $1 \times 10^{-4} M$ | $5 \times 10^{-4} M$ |
|-----------------|---------|----------------------|------------------------|----------------------|----------------------|
| <i>min.</i> | | | | | |
| 20 | 22.3 | 20.6 | 21.5 | 28.6 | 27.8 |
| 40 | 45.3 | 39.0 | 47.5 | 61.4 | 56.8 |
| 60 | 65.5 | 58.2 | 72.8 | 98.9 | 80.5 |
| Per cent change | | -11.1 | +11.6 | +37.3 | +22.9 |

If the Warburg flasks containing the succinoxidase system are allowed to remain stationary, the slow rate of diffusion of oxygen into the reaction medium limits the reoxidation of cytochrome c and consequently the pink color of reduced cytochrome c appears. If, however, the system has been inhibited by α -TPh, the color of the cytochrome c remains that of the oxidized form.

The succinoxidase system is generally considered to consist of succinic dehydrogenase, cytochrome c, and cytochrome oxidase. Hence,

TABLE III

Failure of Ca Added after 20 Min. to Reverse Inhibition of Liver Succinoxidase by $1 \times 10^{-4} M$ α -TPh

| Time | Control | No Ca | $1 \times 10^{-3} M$ Ca |
|---------------------|---------|-------|-------------------------|
| <i>min.</i> | | | |
| 20 | 26.6 | 2.2 | 2.1 |
| 40 | 53.6 | 2.6 | 2.4 |
| 60 | 79.1 | 3.6 | 3.4 |
| Per cent inhibition | | 95.5 | 95.7 |

TABLE IV
*Reversal of α -TPh Inhibition of Liver Succinoxidase
 by Ca Added at Start of Experiment*

| Time | Control | $1 \times 10^{-4} M$ α -TPh | $1 \times 10^{-4} M$ α -TPh $4 \times 10^{-4} M$ Ca |
|---------------------|---------|---------------------------------------|---|
| <i>min.</i> | | | |
| 20 | 22.0 | 7.2 | 22.8 |
| 40 | 43.9 | 9.4 | 43.1 |
| 60 | 63.2 | 11.0 | 58.2 |
| Per cent inhibition | | 82.6 | 7.9 |

the effect of α -TPh on each of these enzymatic links was determined in an effort to localize the action of α -TPh in this system.

It is obvious from determinations of the effect of α -TPh on succinic dehydrogenase (Table V) that while a high concentration of $1 \times 10^{-3} M$ α -TPh causes about 85% inhibition, $1 \times 10^{-4} M$ α -TPh is practically without effect.

To determine the effect of α -TPh on cytochrome c, a system was used in which cytochrome c was reduced nonenzymatically by ascorbic acid and reoxidized by an excess of cytochrome oxidase. This system was obtained by employing the Potter and Schneider assay for cytochrome oxidase using a low cytochrome c concentration (Table VI).

In these experiments it was found that $1 \times 10^{-3} M$ α -TPh almost completely inhibited the nonenzymatic reduction of cytochrome c. By increasing the cytochrome c concentration six-fold (so that it equals the high concentration of cytochrome c employed in the cytochrome oxidase assay) the inhibition was decreased to 62% (Table VI). It seems, therefore, that the inhibiting effect of $1 \times 10^{-3} M$ α -TPh is

TABLE V
The Effect of α -TPh on Liver Succinic Dehydrogenase

| Time | Control | $1 \times 10^{-4} M$ α -TPh | $1 \times 10^{-3} M$ α -TPh |
|---------------------|---------|---------------------------------------|---------------------------------------|
| <i>min.</i> | | | |
| 20 | 7.5 | 7.6 | 1.6 |
| 40 | 14.3 | 13.6 | 2.6 |
| 60 | 20.1 | 19.0 | 2.9 |
| Per cent inhibition | | 5.5 | 85.6 |

on the cytochrome c and not on the cytochrome oxidase. However, at a concentration of $1 \times 10^{-4} M$, α -TPh exerted no effect on the cytochrome oxidase system with either a low or a high cytochrome c concentration (Table VI).

TABLE VI

The Effect of α -TPh on Cytochrome c and Liver Cytochrome Oxidase

| Cytochrome c | Time | Control | $1 \times 10^{-4} M$ α -TPh | $1 \times 10^{-3} M$ α -TPh |
|---------------------------------|------------|---------|---------------------------------------|---------------------------------------|
| 0.2 ml. of $2 \times 10^{-4} M$ | min. 20 | 26.4 | 29.6 | 0 |
| | 40 | 56.4 | 60.6 | 2.8 |
| | 60 | 90.2 | 89.1 | 6.4 |
| Per cent inhibition | | | 1.2 | 92.9 |
| 1.2 ml. of $2 \times 10^{-4} M$ | 20 | 47.0 | 48.6 | 18.2 |
| | 40 | 97.0 | 98.2 | 37.3 |
| | 60 | 154.3 | 151.9 | 58.5 |
| Per cent inhibition | | | 1.6 | 62.1 |

The effect of $1 \times 10^{-3} M$ α -TPh on cytochrome c may easily be seen with the naked eye. If α -TPh is added to cytochrome c which has been oxidized by potassium ferricyanide, the cytochrome c can no longer be reduced with ascorbate, as evidenced by the failure of the pink color of reduced cytochrome c to appear when the reducing agent has been added.

DISCUSSION

Ames (8) concluded that α -TPh causes inhibition of the succinoxidase system by precipitating the calcium which is usually added in the Schneider and Potter (12) assay of succinic dehydrogenase. However, the data of Ames indicate that with a concentration of α -TPh ($4 \times 10^{-4} M$) equal to the calcium concentration prescribed by Schneider and Potter, the system is inhibited 85%, provided the calcium is added before the α -TPh and after the phosphate buffer. It can be calculated that, if this degree of inhibition were entirely due to

the removal of calcium, the activation of the succinoxidase system by calcium should amount to about 570%. Actually, it has been reported to be considerably less (10,14,15). This suggests that a direct effect of α -TPh on the enzymes is exerted.

In our study of the mechanism of inhibition of succinoxidase by α -TPh, calcium was omitted from the assay system since nearly maximal activity could be obtained through "spontaneous activation" by allowing the homogenate to stand in the cold for several hours (9,10). By this simple procedure the confusion resulting from the mutual precipitation of calcium and α -TPh is entirely eliminated. In such systems it is found that α -TPh has a powerful inhibiting effect upon succinoxidase activity, since $1 \times 10^{-4} M$ causes 83-98% inhibition and even $5 \times 10^{-5} M$ may cause very considerable inhibition. Thus, the system without calcium is considerably more sensitive to the action of α -TPh. Ames (8) found that $1 \times 10^{-4} M$ α -TPh is practically without effect in the succinoxidase system containing calcium.

The inhibiting effect of α -TPh on succinoxidase should be exerted on one or more of the enzymes which are usually considered to be components of the system, namely, succinic dehydrogenase, cytochrome c, and cytochrome oxidase. However, α -TPh in a concentration of $1 \times 10^{-4} M$ has little or no effect on any one of these components. The inhibitory effect of 5×10^{-5} to $1 \times 10^{-4} M$ α -TPh on the succinoxidase system cannot, therefore, be accounted for by its action on these enzymes. Since $1 \times 10^{-4} M$ α -TPh had no effect in a system oxidizing ascorbate via cytochrome c and cytochrome oxidase, the action of α -TPh in the complete succinoxidase system would appear to be exerted on some mediator situated between succinic dehydrogenase and cytochrome c. Thus, the failure of the pink color of reduced cytochrome c to appear in stationary flasks containing the succinoxidase system inhibited by $1 \times 10^{-4} M$ α -TPh can be explained. Apparently α -TPh is inhibiting some mediator required for the reduction of cytochrome c.

Cytochrome b is one mediator believed to be operating between succinic dehydrogenase and cytochrome c (16,17). However, since cytochrome b together with succinic dehydrogenase may perhaps partake in the reaction sequence between succinate and methylene blue (16,17), it is unlikely that cytochrome b would be the affected mediator. This is borne out by the fact that $1 \times 10^{-4} M$ α -TPh does not significantly inhibit the system involving methylene blue.

Several investigators have provided evidence for the existence of an additional factor between succinic dehydrogenase and cytochrome c (16-24). It is conceivable that α -TPh might inhibit succinoxidase by acting on this intermediate factor. However, the suggestion may also be considered that α -TPh, because of its detergent property, acts non-specifically on the enzyme system by altering the mutual accessibility of the components of the system (25, 26, 27).

It would appear that the inhibition of succinoxidase by α -TPh is characterized by a reaction between α -TPh and the intermediate factor, which proceeds to completion in about 0.5 hr., the time required to achieve maximum inhibition of the system. Moreover, either the reaction is irreversible or the bond formed between α -TPh and the factor is relatively strong, since even a high concentration of calcium ($1 \times 10^{-3} M$), if added after 20 min., fails to affect the course of the inhibition. However, a much lower concentration of $4 \times 10^{-4} M$ calcium, which is usually employed in the succinoxidase assay, causes considerable reversal of the α -TPh inhibition if it is added to the reaction mixture just before the homogenate, which is the last to be added. Apparently sufficient α -TPh is precipitated by the calcium to prevent its inhibiting function.

The failure of α -TPh to inhibit succinoxidase, when it is added directly to the homogenate itself to give a concentration of $1 \times 10^{-4} M$, suggests that it reacts with proteins. Since the concentration of proteins in the homogenate is naturally much greater than in the final reaction mixture, a reaction between α -TPh and proteins in the homogenate would effectively reduce the concentration of α -TPh to the point where inhibition of succinoxidase does not occur.

The ability of a high concentration ($1 \times 10^{-3} M$) of α -TPh to inhibit strongly cytochrome c and succinic dehydrogenase is very interesting. Together with azide (28) and cyanide (29), α -TPh must be recognized as one of the few cytochrome c inhibitors.

The stimulation of the succinoxidase system with very low concentrations of α -TPh may result from the removal of some heavy metal inhibitor which combines with α -TPh. In this respect, the action of α -TPh would be similar to that of aluminum, which appears to stimulate succinoxidase activity by preventing the toxic action of some heavy metal impurity (12).

SUMMARY

The effect of α -TPh on succinoxidase has been studied in a system in which calcium and aluminum were omitted and nearly maximal activity was achieved through "spontaneous activation." Thus, a study of the direct effect of α -TPh on succinoxidase was made possible.

α -TPh strongly inhibits this system, a concentration of $1 \times 10^{-4} M$ exerting 83–98% inhibition. This concentration of α -TPh, however, has little or no effect on succinic dehydrogenase, cytochrome c, or cytochrome oxidase. It is concluded that α -TPh inhibits the succinoxidase system by an action on some unidentified mediator which possibly operates between cytochrome b and cytochrome c. At a high concentration of α -TPh ($1 \times 10^{-3} M$), however, cytochrome c and succinic dehydrogenase are strongly inhibited.

The inhibition of the succinoxidase system by $1 \times 10^{-4} M$ α -TPh reaches a maximum in about 0.5 hr. and after this period of time the inhibition can no longer be reversed with calcium. However, $4 \times 10^{-4} M$ calcium effects considerable reversal of the inhibition provided calcium is added prior to the homogenate, which is the last to be added. If the homogenate itself is made $1 \times 10^{-4} M$ with respect to α -TPh and is allowed to stand at room temperature for 0.5 hr., the succinoxidase system is not inhibited.

Very low concentrations of α -TPh ($2.5 \times 10^{-5} M$ to $5 \times 10^{-6} M$) stimulate the succinoxidase system.

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Enzyme Studies on Rabbits with Incipient Muscle Dystrophy

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INTRODUCTION

The greatly increased oxygen consumption of muscles from vitamin-E-deficient animals prompted investigation of the enzymatic activity of dystrophied muscles (1-7). In vitamin-E-deficient young rats this condition has been observed in muscles even before histological changes were apparent and it must, therefore, be attributed to alterations in the metabolic activity of the muscle fibers themselves (8). Accordingly, enzyme assays should be performed preferably on muscles showing little or no histological changes so as to avoid complications due to replacement of muscle fibers by appreciable fat and fibrous tissue. Hummel (4) found decreased adenosinetriphosphatase activity in dystrophic muscles and attributed this, at least in part, to the replacement of normal muscle by necrotic and fibrous tissue. On the other hand, Basinski and Hummel (3) found normal succinic dehydrogenase activity in dystrophic hamster muscles. By the same reasoning, this could be interpreted as indicating an increased succinic dehydrogenase activity in the unaffected muscle mass. Our investigation of enzyme activity during the early stage of nutritional muscle dystrophy was planned with the purpose in mind to minimize as far as possible this complicating and confusing condition.

EXPERIMENTAL

Two groups each of four young rabbits (weight range: 423-700 g. and 386-758 g., respectively) were used. Nutritional muscle dystrophy was produced in one group on the Eppstein-Morgulis diet (9) in which 10 parts of cod liver oil were substituted for the 10 parts of olive oil. The control group received the same diet plus 12.5 mg. of α -tocopherol acetate every fourth day. Body weight was determined daily as was

also the urinary creatine ratio (9). This ratio was determined in urine samples squeezed out by gentle downward pressure on the abdomen of the rabbit. This ratio serves as a reliable index of the development of dystrophy. The rabbits were killed after about 3 weeks and gastrocnemius muscle and liver were removed and homogenized for the enzyme assays. Pieces of muscle were also fixed for sectioning and the state of dystrophy graded from stained histological preparations. We wish to express our indebtedness to the Department of Pathology and to Prof. J. P. Tollman for these histological examinations.

The activity of the following muscle enzymes was determined: malic dehydrogenase (10), cytochrome oxidase (11), succinic dehydrogenase (11), adenosinetriphosphatase (ATPase) (12). Lactic dehydrogenase and fumarase were determined by replacing malate in the malic oxidase system with equivalent amounts of lactate or fumarate, respectively. Liver uricase (13) and ATPase (12) activities were also measured. Other pertinent experimental details have been previously described (14,15).

RESULTS AND DISCUSSION

The rabbits on the vitamin-E-deficient diet showed no clinical signs of dystrophy. Nevertheless, incipient dystrophy was evidenced by the fact that mild fibrotic changes had already occurred, though not sufficiently to affect the enzyme assays expressed on the basis of dry weight of muscle. The creatine ratio had increased from an average of 28 to an average of 59, thus confirming that the dystrophic process was progressing.

TABLE I

Enzyme Activity in Gastrocnemius Muscle and Liver from Dystrophic Rabbits

| Rabbit number | α -Tocopherol acetate in diet | U_{O_2} at 60 min. ^a | | | | | | P liberated in 15 minutes/mg. wet weight | |
|---------------|--------------------------------------|-----------------------------------|-----------------------------|---------------------------------|-------------------------------|-------------------|-----------------|--|----------------|
| | | Malic dehydrogenase (muscle) | Cytochrome oxidase (muscle) | Succinic dehydrogenase (muscle) | Lactic dehydrogenase (muscle) | Fumarase (muscle) | Uricase (liver) | ATPase (muscle) | ATPase (liver) |
| 1 | + | 21.1 | 179.5 | 21.7 | 12.3 | 16.7 | 2.6 | 17.8 | 12.5 |
| 2 | + | 30.6 | 171.6 | 29.5 | 16.9 | 24.8 | 2.5 | 14.6 | 6.5 |
| 3 | + | 14.1 | 95.7 | 13.4 | 7.2 | 13.0 | 2.5 | 19.2 | 7.8 |
| 4 | + | 19.8 | 162.8 | 20.2 | 9.7 | 18.6 | 3.0 | 16.9 | 8.8 |
| 5 | — | 29.2 | 182.8 | 30.5 | 15.9 | 23.9 | 3.5 | 14.7 | 7.1 |
| 6 | — | 20.9 | 187.6 | 25.7 | 12.5 | 19.5 | 3.0 | 9.6 | 6.4 |
| 7 | — | 14.4 | 149.4 | 15.3 | 9.6 | 14.2 | 4.0 | 13.4 | 9.8 |
| 8 | — | 25.1 | 160.3 | 26.0 | 13.1 | 20.7 | 4.5 | 17.0 | 10.8 |

^a U_{O_2} represents the cubic millimeters of O_2 consumed/mg. dry weight of tissue in the time, t.

The results of these assays are summarized in Table I. It is apparent that no significant alteration has occurred in the activity of the various enzymes in muscle or liver homogenates from dystrophic rabbits. The assay systems used in this study are designed to measure the maximum possible activity since there is present an excess of substrate and required cofactors for each enzyme tested. But the assays do not reflect the actual enzyme activity as it occurs *in vivo*. It appears from this work with tissue homogenates that the enzyme spectrum of the active muscle mass is practically the same in dystrophic and normal muscle. The enhanced oxidative activity of dystrophied muscle would seem, therefore, to stem from a defect in the intrinsic control of enzyme activity rather than from altered enzymic concentrations. This is also borne out by the fact that the high oxygen uptake is dependent upon structural integrity of muscle (1).

SUMMARY

Malic dehydrogenase, cytochrome oxidase, succinic dehydrogenase, lactic dehydrogenase, fumarase, and adenosinetriphosphatase activities in muscle (gastrocnemius) homogenates from rabbits with incipient nutritional muscle dystrophy are the same as in homogenates from normal animals. The same is true for uricase and adenosinetriphosphatase activities of liver homogenates from normal or dystrophic rabbits.

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Effect of α -Tocopheryl Phosphate on Enzyme Activity

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INTRODUCTION

Houchin (1) was the first to observe an inhibition of succinoxidase by α -tocopheryl phosphate (α -TPh). This finding has since been confirmed by several investigators (2-6). We observed that with a "spontaneously activated" succinoxidase system from which calcium and aluminum were omitted, even as low as $5 \times 10^{-5} M$ α -TPh produced inhibition (5). This could be accounted for by an action of α -TPh on some unidentified mediator functioning between succinic dehydrogenase and cytochrome c. Moreover, with this system, very low concentrations of α -TPh ($2.5 \times 10^{-5} M$ to $5 \times 10^{-6} M$) actually produced stimulation. In view of the marked sensitivity of succinoxidase to α -TPh, it was of interest to study the effect of α -TPh on other enzymes (7-12).

EXPERIMENTAL

Organs from adult albino rats were treated as described previously (5). All solutions were prepared with glass-redistilled water and adjusted to pH 7.4 wherever necessary. Two samples of α -TPh were used in these experiments, one synthesized in this laboratory and another kindly furnished by Hoffmann-La Roche, Inc. Cytochrome c was prepared from beef or horse hearts by the method of Keilin and Hartree (13) and dialyzed against glass-redistilled water. Malic oxidase activity was measured by Potter's method (14) and also by replacing the cytochrome c in Potter's system with 0.2 ml. of 0.5% methylene blue. Lactic oxidase activity was measured by replacing the malate in the malic oxidase system employing methylene blue with an equivalent amount of lactate. D-Amino acid oxidase activity was measured by the procedure of Axelrod *et al.* (15), as was also uricase activity (16). Cholinesterase activity was measured by the procedure of Stadie *et al.* (17). The gas phase was 5% CO₂ and 95% O₂. Since the oxygen consumption of this system is negligible, the oxygen in the gas phase does not interfere with the determination. The method of DuBois and Potter (18) was used to measure adenosinetriphosphatase activity. Catalase activity was

measured by the method of Kreke *et al.* (19). Liver acid phosphatase activity was measured by incorporating features of several methods (20,21,22). The reaction mixture consisted of 0.5 ml. of 0.5 *M* acetate buffer, pH 5; 0.5 ml. of 1.5% glycerophosphate (mixture of the alpha and beta forms), pH 5; 2 ml. of water; 0.5 ml. of 10% liver homogenate; and 2 drops of toluene as a preservative. The tubes were incubated for 4 hr. at 38°C. The reaction was stopped by adding 1.5 ml. of 20% trichloroacetic acid, the contents filtered, and phosphorus determined in suitable aliquots by the method of Fiske and SubbaRow (23).

RESULTS AND DISCUSSION

α -TPh in a concentration of 1×10^{-3} *M* strongly inhibits the malic oxidase system employing cytochrome c (Table I). Since we have observed previously that at this concentration α -TPh will strongly inhibit cytochrome c (5), the inhibition of the malic oxidase system may also be attributed to this effect. This is borne out by the fact that the system is only slightly inhibited by 1×10^{-3} *M* α -TPh when the cytochrome c is replaced by methylene blue (Table I). However, α -TPh in a concentration of 1×10^{-4} *M* has only a slight inhibitory effect on either of the malic oxidase systems (Table I). This observation would suggest that the virtually complete inhibition of succinoxidase by 1×10^{-4} *M* α -TPh, which we have observed (5), is not caused by a nonspecific detergent action of α -TPh. If 1×10^{-4} *M* α -TPh inhibited the succinoxidase system through its detergent property by altering the mutual accessibility of the components of the system, one should expect a similar high degree of inhibition in the malic oxidase-cytochrome c system. The failure of any significant inhibition thus strengthens the view that α -TPh inhibits succinoxidase by a direct action on an unidentified mediator required for the reduction of cytochrome c. The malic oxidase system with cytochrome c devised by Potter requires for its activity the operation of the following enzymes: malic dehydrogenase, coenzyme I-cytochrome c reductase, transaminase, cytochrome c, and cytochrome oxidase. Since 1×10^{-4} *M* α -TPh has little effect on this system, it follows that α -TPh at that concentration would be essentially without effect on each of the component enzymes. Since 1×10^{-3} *M* α -TPh has only a slight inhibitory action on the malic oxidase system with methylene blue, even that concentration of α -TPh would be without appreciable effect on malic dehydrogenase, coenzyme I-cytochrome c reductase, and transaminase.

In the lactic oxidase system with methylene blue, 1×10^{-3} *M* and 1×10^{-4} *M* α -TPh exert inhibitions of 17% and 13%, respectively

(Table I). Thus, α -TPh is without marked effect in this system also. α -TPh was without effect on both D-amino acid oxidase and uricase (Table I). Cholinesterase was inhibited 29% by $1 \times 10^{-3} M$ α -TPh, whereas $1 \times 10^{-4} M$ α -TPh had no effect (Table I).

Adenosinetriphosphatase activity in both muscle and lung was unaffected by the presence of α -TPh. The reaction mixtures contained 0.2 ml. of 2% muscle or liver homogenate (Table II). Since calcium is present in the assay system in excess of the α -TPh, the α -TPh would

TABLE I
The Effect of α -Tocopheryl Phosphate on Several Enzymes

| | UO_2 at the time indicated ^a | | | | | |
|--|---|---------------------------------|---------------------------------------|----------|---------------------------------------|----------|
| | Time O_2 | Without α -TPh t | $1 \times 10^{-4} M$ α -TPh | | $1 \times 10^{-3} M$ α -TPh | |
| | | | O_2 | Change | O_2 | Change |
| | min. | cu. mm. | cu. mm. | per cent | cu. mm. | per cent |
| Malic oxidase with cytochrome c (liver) | 55 | 112.2 | 101.5 | -10 | 31.5 | -72 |
| Malic oxidase with methylene blue (liver) | 55 | 89.3 | 82.2 | - 8 | 79.7 | -11 |
| Lactic oxidase with methylene blue (liver) | 60 | 32.4 | 28.3 | -13 | 26.8 | -17 |
| D-Amino acid oxidase (kidney) | 60 | 18.2 | | | 19.0 | + 4 |
| Uricase (liver) | 60 | 11.4 | 11.7 | + 3 | 12.6 | +11 |
| Cholinesterase (brain) | 60 | 29.1 | 29.6 | + 2 | 20.6 | -29 |

^a UO_2 is the cubic millimeters O_2 consumed/mg. dry weight of tissue in the time t . For cholinesterase, the values represent cubic millimeters CO_2 liberated/mg. dry weight of brain in 60 min.

be precipitated as the insoluble calcium salt. The calcium remaining in solution was apparently still sufficient for maximum activation of the system. Carey and Dziewiatkowski (11) have observed that if calcium is omitted from the system, α -TPh inhibits adenosinetriphosphatase. It is thus apparent that alkaline-earth metals must not be present in an enzyme system if a direct effect of α -TPh upon the enzyme is to be demonstrated^(5,24). Carey and Dziewiatkowski (11) have proposed, however, that the inhibition, which they observed in the system to which no calcium was added, was due to a removal of the

calcium originally present in the homogenate by α -TPh. This possibility is unlikely in view of the solubility data presented by Ames (4) which indicate that the solubility product of calcium α -tocopheryl phosphate could not possibly have been exceeded in the absence of added calcium.

The increased activity of catalase in the presence of $1 \times 10^{-3} M$ α -TPh is an interesting observation (Table III). Increasing the concentration of α -TPh to $2 \times 10^{-3} M$ caused no further increase in activity. α -TPh, itself, had no action on hydrogen peroxide under the

TABLE II

The Effect of α -Tocopheryl Phosphate on Adenosinetriphosphatase Activity

| Tissue | α -TPh | P liberated in system in 15 min. | Average |
|--------|--------------------|--|---------|
| Muscle | <i>M</i> | <i>μ</i> | |
| | 0 | 66.4 | |
| | 0 | 69.8 | 68.1 |
| | 5×10^{-4} | 69.0 | 69.0 |
| Muscle | 0 | 72.2 | |
| | 0 | 69.9 | 71.0 |
| | 1×10^{-3} | 75.6 | |
| | 1×10^{-3} | 76.7 | 76.1 |
| Lung | 0 | 49.8 | |
| | 0 | 48.2 | 49.0 |
| | 5×10^{-4} | 50.1 | |
| | 5×10^{-4} | 51.0 | 50.6 |

conditions of the test. Possibly catalase exerts a peroxidatic action on α -TPh.

α -TPh in a concentration of $1 \times 10^{-3} M$ inhibited liver acid phosphatase only very slightly, whereas $1 \times 10^{-4} M$ α -TPh was without effect. Carey and Dziewiatkowski (11) have reported an inhibition of acid phosphatase by α -TPh. However, these investigators employed rabbit muscle homogenates and used phenyl phosphate as the substrate. It is possible that the differences in the set up of these experiments may account for the difference in the results obtained.

It is apparent from this study that the *in vitro* effects of α -TPh

TABLE III

The Effect of α -Tocopheryl Phosphate on Catalase Activity

| α -TPh | Decomposition of H_2O_2 |
|--------------------|---|
| <i>M</i> | <i>per cent</i> |
| 0 | 58.2 |
| 0 | 47.4 |
| 0 | 45.0 |
| | 50.2 (Av.) |
| 1×10^{-3} | 65.4 |
| 1×10^{-3} | 65.2 |
| 1×10^{-3} | 66.3 |
| | 65.6 (Av.) |
| 2×10^{-3} | 64.8 |
| 2×10^{-3} | 65.1 |
| 2×10^{-3} | 64.7 |
| | 64.9 (Av.) |

probably cannot be attributed to its surface activity. The nine enzyme systems studied, representing a total of eleven enzymes, exhibited little or no inhibition in the presence of 1×10^{-4} *M* α -TPh or in some cases even 1×10^{-3} *M* α -TPh. Catalase, moreover, showed definite activation in 1×10^{-3} *M* α -TPh. It would seem, therefore, unlikely

TABLE IV

The Effect of α -Tocopheryl Phosphate on Liver Acid Phosphatase Activity

| α -TPh | P liberated in system in 4 hr. |
|--------------------|--------------------------------------|
| <i>M</i> | <i>μg.</i> |
| 0 | 495 |
| 0 | 460 |
| | 478 (Av.) |
| 1×10^{-3} | 437 |
| 1×10^{-3} | 452 |
| | 445 (Av.) |
| 1×10^{-4} | 491 |
| 1×10^{-4} | 468 |
| | 480 (Av.) |

that the marked repression of succinoxidase activity by 1×10^{-4} M α -TPh, which was not observed with other enzyme systems, could be due to a nonspecific protein denaturation by a surface active agent.

SUMMARY

The activity of a number of enzyme systems in the presence of α -TPh has been determined.

α -TPh in a concentration of 1×10^{-3} M markedly inhibits a malic oxidase system employing cytochrome c and cholinesterase; it has little or no effect on a malic oxidase-methylene blue system, a lactic oxidase-methylene blue system, D-amino acid oxidase, uricase, an adenosinetriphosphatase system containing calcium, and liver acid phosphatase. In a concentration of 1×10^{-4} M , α -TPh has little or no effect on any of these systems.

α -TPh in a concentration of 1×10^{-3} M definitely increases catalase activity.

In view of these results it is suggested that the marked inhibition of succinoxidase by 1×10^{-4} M α -TPh cannot be attributed to a non-specific detergent action.

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Yeast Nucleic Acid. III. The Effect of Glycine on Yeast Proliferation and Nucleic Acid Biosynthesis

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INTRODUCTION

It has been demonstrated that different strains of yeast propagated under identical conditions vary in their ability to synthesize nucleic acids and proteins (1). Abrams, Hammarsten, and Shemin (2) propagated *Torula utilis* yeast on tagged glycine for 1 hr. and concluded that some glycine entered directly the 4, 5, and 7 positions of guanine. Schultz and Pomper (3) previously reported the ability of this yeast to utilize the nitrogen of glycine for extensive cell proliferation. It was considered of interest to investigate the effect of glycine upon the synthesis of nucleic acids by a yeast incapable of using it for cell proliferation and also to record the effects upon nucleic acid synthesis of amino acids not utilizable for proliferation by *Torula utilis*.

Torula utilis (Northern Regional Research Laboratory) produced good crops on either glycine or lysine as the only source of nitrogen, but failed to proliferate when furnished histidine as the sole nitrogen source (3). The other yeast selected for this work was a commercial bakers' yeast, *Saccharomyces cerevisiae* Hansen, requiring pantothenic acid and biotin as growth factors (4). This yeast was incapable of proliferation in a medium containing glycine, lysine, or histidine as the only nitrogenous component (3).

EXPERIMENTAL

Propagation of Yeasts on Molasses Supplemented with Amino Acids

Seed yeasts were obtained by propagation of pure yeast cultures on 10 Balling molasses wort adequately supplemented with ammonium and phosphate ions. The moist seed yeast (100 g.) was suspended in 4.5 l. of water to which 3.0 g. of phosphoric acid and the quantities of ammonium sulfate, glycine, lysine, and histidine indicated

in Table I were added. Molasses (300 g.) was added by the continuous process described by Hayduck (5) at a rate increasing hourly by 1.2. The wort was aerated at the rate of 28 times the volume of the medium per minute during the 8-hr. propagation period, and the pH was maintained within the range of 4.8–5.5 by adding sodium carbonate as required. The yeast was separated from the spent wort and washed by centrifugation.

Propagation of Yeasts on Synthetic Media

The seed was 60 g. of moist yeast. This was propagated in the manner described above on a medium consisting of 150 g. of sucrose, 3.0 g. of magnesium sulfate heptahydrate, 1.0 g. of calcium chloride dihydrate, 3.0 g. of potassium chloride, 360 mg. of inositol, 13.5 mg. of calcium pantothenate, and 0.18 mg. of biotin.

Nucleic Acid Analyses

The nucleic acid determinations were performed by the spectrophotometric method reported previously (6).

RESULTS AND DISCUSSION

The differences in character of the two yeasts investigated were sharp (Table I). *Torula utilis* proliferated almost as extensively on glycine and lysine as it did on ammonium sulfate, and it utilized the nitrogen of these amino acids for the biosynthesis of nucleic acids. The total nucleic acid contents of *Torula utilis* propagated on ammonium sulfate, glycine, or lysine were very similar. Histidine, on the other hand, was not used for cell proliferation and had no effect upon the nucleic acid content of *Torula utilis*.

The *Saccharomyces cerevisiae* was unable to utilize any of the three amino acids for appreciable proliferation. Yet the seed yeast provided with glycine synthesized nearly five times as much nucleic acid as did the control yeast. This finding suggests the conversion of glycine into nucleic acids by some direct mechanism and is in accord with the conclusion drawn by Abrams, Hammarsten, and Shemin (2) that glycine is a nucleic acid building block. In their work with *Torula utilis* more than twice as much glycine nitrogen was found to enter the 7 position than any other in guanine. It is believed that this ratio is considerably higher with the *Saccharomyces cerevisiae* strain employed in our work wherein the synthesis of nucleic acids was augmented with some specificity and not simply with concurrent cell proliferation.

The synthesis of nucleic acids has been associated frequently with rapidly proliferating cells; e.g., Caspersson and his co-workers demonstrated that cells of kidney, liver, intestine (7), plants (8,9), and

insects (8) which are dividing rapidly have far greater nucleic acid contents than corresponding resting or senescent cells. In yeast it was shown that a high concentration of nucleic acid is correlated with growth and not merely with metabolic activity; thus a culture which was fermenting rapidly but not growing was found to have a relatively low nucleic acid content, whereas rapid proliferation was accompanied

TABLE I
Yeasts Propagated on Molasses^a Supplemented with Amino Acids

| Culture | (NH ₄) ₂ SO ₄ | Amino acid ^d | New yeast, dry weight | N ^e | Nucleic acid ^f | Nucleic acid synthesized |
|--|---|-------------------------|-----------------------|----------------|---------------------------|--------------------------|
| | g. | | g. | per cent | per cent | g. |
| <i>Torula utilis</i> ^b | — | — | 46.3 | 5.05 | 2.00 | 0.93 |
| <i>Torula utilis</i> ^b | 30 | — | 85.0 | 7.65 | 3.72 | 3.58 |
| <i>Torula utilis</i> ^b | — | Glycine ^c | 74.7 | 8.14 | 4.02 | 3.51 |
| <i>Torula utilis</i> ^b | — | Lysine ^c | 74.9 | 7.63 | 3.95 | 3.43 |
| <i>Torula utilis</i> ^b | — | Histidine ^f | 39.7 | 8.30 | 2.21 | 0.93 |
| <i>S. cerevisiae</i> Hansen ^c | — | — | 44.4 | 5.05 | 3.28 | 0.44 |
| <i>S. cerevisiae</i> Hansen ^c | 30 | — | 75.2 | 8.02 | 5.30 | 3.53 |
| <i>S. cerevisiae</i> Hansen ^c | 30 | Glycine ^f | 72.9 | 8.76 | 5.95 | 4.07 |
| <i>S. cerevisiae</i> Hansen ^c | — | Glycine ^f | 45.4 | 8.17 | 5.54 | 2.12 |
| <i>S. cerevisiae</i> Hansen ^c | — | Lysine ^f | 27.6 | 7.34 | 4.63 | 0.63 |
| <i>S. cerevisiae</i> Hansen ^c | — | Histidine ^f | 47.0 | 8.08 | 3.30 | 0.53 |

^a Three hundred g. of molasses contained 0.997% or 2.99 g. of N.

^b Seed yeast had dry weight of 24.2 g., and contained 7.01% N and 2.00% nucleic acid.

^c Seed yeast had dry weight of 27.1 g., and contained 8.08% N and 7.15% nucleic acid.

^d The weights of amino acids employed were: 34.1 g. glycine; 82.8 g. L-lysine monohydrochloride; 86.9 g. L-histidine monohydrochloride.

^e Utilizable for growth by this yeast in absence of other sources of N.

^f Not utilizable for growth by this yeast in absence of other sources of N.

^g Calculated to a dry basis.

by high levels of nucleic acid (6,9,10). Therefore it is considered interesting to note the synthesis of nucleic acids and proteins by the cells of *S. cerevisiae* undergoing proliferation slowly.

S. cerevisiae propagated on molasses supplemented with glycine converted approximately half of the glycine nitrogen into proteins (Table I). The failure of this organism to proliferate extensively on this

TABLE II
Yeasts Propagated on Synthetic Media

| Culture | (NH ₄) ₂ SO ₄ | Glycine | New yeast, dry weight | N ^d | Nucleic acid ^d | Nucleic acid synthesized |
|--|---|-------------------|-----------------------|----------------|---------------------------|--------------------------|
| | g. | g. | g. | per cent | per cent | g. |
| <i>Torula utilis</i> ^a | 35 | — | 70.6 | 6.88 | 4.17 | 3.25 |
| <i>Torula utilis</i> ^a | 5 | — | 35.7 | 4.06 | 1.68 | 0.55 |
| <i>Torula utilis</i> ^a | 5 | 34.1 ^c | 72.9 | 7.71 | 4.40 | 3.55 |
| <i>S. cerevisiae</i> Hansen ^b | 35 | — | 64.1 | 8.14 | 6.85 | 4.35 |
| <i>S. cerevisiae</i> Hansen ^b | 5 | — | 41.4 | 4.12 | 3.23 | 0.70 |
| <i>S. cerevisiae</i> Hansen ^b | 5 | 34.1 | 32.2 | 8.25 | 4.86 | 1.20 |

^a Seed yeast had dry weight of 14.52 g., and contained 7.01% N and 2.00% nucleic acid.

^b Seed yeast had dry weight of 16.26 g., and contained 8.08% N and 7.15% nucleic acid.

^c Equivalent in N content to 30 g. of (NH₄)₂SO₄.

^d Calculated to a dry basis.

medium may be attributable to the formation of a residue inhibitory to the yeast as the result of the utilization of glycine nitrogen. An alternative explanation is that the yeast, known to contain a great preponderance of cytoplasmic ribonucleic acid (RNA) over nuclear

TABLE III
Rate of Nucleic Acid Synthesis from Glycine by S. cerevisiae Hansen^a on Molasses^b in Absence of Added Ammonia

| Glycine | Fermentation time | New yeast, dry weight | N ^c | Nucleic acid ^c | Nucleic acid synthesized |
|---------|-------------------|-----------------------|----------------|---------------------------|--------------------------|
| g. | hr. | g. | per cent | per cent | g. |
| — | 3 | 12.1 | 6.66 | 3.35 | 0.11 |
| — | 5 | 21.8 | 5.90 | 2.72 | 0.18 |
| — | 8 | 36.3 | 5.07 | 1.94 | 0.15 |
| 15 | 3 | 7.6 | 9.06 | 5.34 | 0.48 |
| 15 | 5 | 14.7 | 8.49 | 5.07 | 0.77 |
| 15 | 8 | 30.3 | 8.37 | 4.67 | 1.36 |

^a Seed yeast had dry weight of 18.4 g., and contained 8.40% N and 4.95% nucleic acid.

^b Three hundred g. of molasses contained 0.997% or 2.99 g. of N.

^c Calculated to a dry basis.

desoxypentosenucleic acid (DNA), may be so retarded in its synthesis of DNA that it is not capable of continued cell proliferation.

Torula utilis readily utilized glycine for cell proliferation and nucleic acid production in the absence of molasses (Table II). *S. cerevisiae* was incapable of extensive nucleic acid biosynthesis under the same conditions.

It is evident from the data presented in Table III that the yeast furnished neither ammonia nor glycine multiplied slowly. Its nucleic acid and protein levels decreased during fermentation. Very little nucleic acid was synthesized, and protein synthesis was slow. To a large measure, the nucleic acid and proteins of the seed yeast were shared with new cells.

The yeast furnished glycine proliferated at a slower rate than did the yeast supplied no glycine. Despite this condition, appreciable and sustained syntheses of nucleic acid and protein occurred.

The high nitrogen contents of yeasts propagated on histidine (Table I) suggest that this amino acid served as a nitrogen source for protein synthesis.

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SUMMARY

1. *Torula utilis* utilized glycine and lysine for cell proliferation and for the biosynthesis of nucleic acids. Histidine was used neither for proliferation nor for nucleic acid synthesis.

2. *Saccharomyces cerevisiae* Hansen utilized glycine for nucleic acid biosynthesis, but not for proliferation. Lysine and histidine served neither function.

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The Effect of Cobalt on the Activity of Certain Enzymes in Homogenates of Rat Tissue ¹

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INTRODUCTION

Studies of the effect of cobaltous ions on the respiration of slices of animal tissues have been reported by Burk *et al.* (1,2). Cobalt in a concentration of approximately $1 \times 10^{-3} M$ caused an inhibition of the endogenous respiration of a number of tissues, which inhibition was progressive with time of contact of cobalt with the tissue. Addition of histidine to the cobalt-treated tissues stopped the increase in inhibition, but did not cause the respiratory level to revert to that of an uninhibited control tissue. The action of cobalt on the respiration of animal tissue slices differed from its action on intact bacteria, specifically *Proteus vulgaris* (3). Under experimental conditions similar to those used in the animal tissue studies, the endogenous respiratory rate of these microorganisms is not affected by cobalt.

To determine whether or not the inhibition of respiration observed with slices of animal tissues was attributable to the effect of cobalt on a specific enzyme system, we tested certain enzymes in homogenates of rat liver and kidney for sensitivity to cobalt. Both oxidative and non-oxidative, as well as -SH-dependent and -SH-independent, enzymes were included in this study. We tested the -SH-dependent enzymes for cobalt inhibition under both aerobic and anaerobic conditions to establish whether an observed inhibition might be due to a mercaptide of the type $(RS)_2Co$ whose formation should be independent of the presence of oxygen.

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EXPERIMENTAL

In all experiments, homogenates of rat liver or kidney were used as the source of enzyme studied. Except where noted, the tissue was homogenized in ice water with a glass homogenizer and kept cold until used. $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ was added to the homogenate 5–15 min. before addition of substrate, to give a final concentration of $1 \times 10^{-3} M$. Many of the usual procedures for enzyme assay involve the use of relatively high concentrations of phosphate buffer. Since cobalt phosphate is precipitated at physiological pH's in the presence of much phosphate, barbiturate buffer, or acetate buffer, Ringer's solution either lightly buffered with phosphate or containing no phosphate was substituted.

Anaerobic dehydrogenase activities were determined by the Thunberg technique (6). Methylene blue served as the indicator for the oxidation of succinate and of malate, while *o*-chlorphenol-indophenol was used as the indicator for choline and lactate oxidation. Aerobic and anaerobic succinic dehydrogenase activity was also measured using triphenyltetrazolium chloride as hydrogen acceptor (17). Anaerobic phosphatase activities were determined in Warburg vessels filled with nitrogen, and are expressed as micrograms of inorganic phosphorus liberated by the indicated amount of tissue in the given time. More specific methods for the separate enzymes are indicated below.

Adenosine triphosphate and coenzyme I were prepared by the methods of LePage (4,5). Cytochrome c was a commercial preparation (Tremond Chemical Co., N. Y.).

At least three separate experiments were performed for each of the enzyme assays reported in this paper.

Succinoxidase

The activity of succinoxidase has been reported to depend on intact -SH groups (7). The measurements of its activity under aerobic conditions was based on the method of Schneider and Potter (8).

Choline Oxidase

The measurement of the activity of choline oxidase under aerobic conditions was based on the method of Mann and Quastel (9). This enzyme has been considered to be -SH-dependent (10).

Adenosine Triphosphatase

It has been reported that the activity of adenosinetriphosphatase is dependent on intact -SH groups in its molecule (11). The activity of this enzyme was measured by the method of Dubois and Potter (12).

The determinations were carried out aerobically and anaerobically in Warburg vessels. The inorganic phosphorus was determined by the method of Fiske and SubbaRow (13).

Malic Oxidase-Cytochrome Reductase System

Malic oxidase has been reported to be dependent on -SH groups for its activity while cytochrome reductase is not (11). The activity of this system was measured manometrically by the method described in Umbreit *et al.* (12).

The determination of the effect of cobalt on malic dehydrogenase under anaerobic conditions was made in Thunberg tubes. The reaction mixture was the same as that used aerobically for assay of the malic oxidase system except that methylene blue replaced the cytochrome system as the hydrogen acceptor. Cobalt was not added to the tissue homogenate until after the tubes had been evacuated.

Cytochrome Oxidase

Cytochrome oxidase reportedly is not dependent for its activity on intact -SH groups in its molecule (11). The method of Schneider and Potter (8) was employed for the measurement of the activity of this enzyme in the presence and absence of cobalt.

β -Glycerophosphatase

The activity of β -glycerophosphatase is not dependent on the presence of intact -SH groups (11). The sensitivity of this enzyme to cobalt was tested as follows: Tubes containing the reaction mixture were incubated at 37.5°C. for 1 hr. with stirring at 5-min. intervals. Each tube contained: 0.2 or 0.3 ml. of 5% rat liver or kidney homogenate, 2.0 ml. of 0.1 *M* acetate buffer, pH 5.5 or pH 6.2, 1.0 ml. (≈ 7.76 mg.) sodium β -glycerophosphate and 0.1 ml. water or 0.1 ml. cobalt. The volume was made up to 4.0 ml. with water. The reaction was stopped at the end of 1 hr. by the addition of 0.5 ml. 50% trichloroacetic acid. Inorganic phosphorus was determined on aliquots of the supernatant fluid by the method of Fiske and SubbaRow (13).

Lactic Oxidase System

The activity of lactic oxidase has been reported to be independent of -SH groups (11). The method used for the determination of the lactic oxidase system was that described for the measurement of the activity of the malic oxidase system, except that 0.3 ml. of 1 *M* sodium lactate, pH 7.0 was used in place of sodium malate.

The effect of cobalt on lactic dehydrogenase under anaerobic conditions was determined with o-chlorophenol indophenol as hydrogen acceptor in a reaction mixture otherwise similar to that used in the aerobic study.

Catalase

The presence of intact -SH groups is not essential to the activity of catalase (11). Determination of the activity of this enzyme in the presence or absence of cobalt was made by the method of von Euler and Josephson (14).

Nucleotidase

This enzyme, which effects dephosphorylation of nucleic acids, has not been characterized for -SH-dependence.

The nucleotidase activity in rat kidney homogenates and the effect of $1 \times 10^{-3} M$ cobalt thereon was determined by the amount of inorganic phosphorus liberated from yeast ribonucleic acid added to the homogenates. Liver homogenates were not tested because of liver's low nucleotidase activity (15).

The assays were carried out as follows: into 15-ml. conical centrifuge tubes were placed 0.4 or 0.6 ml. of a 5% rat kidney homogenate in physiological saline, 1.3 or 1.1 ml. of water or of a cobalt solution, and then 1 ml. of yeast ribonucleic acid solution (≈ 5 mg.) at pH 7.4. After the tubes were incubated at 37.5°C . for 1 hr. with stirring at 5-min. intervals, the reaction was stopped by the addition of 0.3 ml. of 100% trichloroacetic acid to each of the tubes, which then were shaken well and centrifuged. Three milliliters of absolute ethyl alcohol was added to 1-ml. aliquots of the supernatant to precipitate the remaining nucleic acid. Aliquots of the supernatant were removed for inorganic phosphorus determinations by the method of Fiske and SubbaRow (13). Known amounts of inorganic phosphorus were treated in a manner similar to the experimental samples in order to obtain a standard phosphorus curve.

RESULTS

The results are summarized in the three tables below. Table I *A* gives the results obtained with manometric measurements; Table I *B* the results obtained with Thunberg tubes under anaerobic conditions; Table II the results obtained with phosphorylytic enzymes; and Table III with catalase.

The cytochrome oxidase activity of a tissue, as determined by conventional methods, is considered by some to be in excess over any one of the dehydrogenating enzymes, although others have not found this to be true. In our hands, the Q_{O_2} for cytochrome oxidase was consistently only slightly higher than the Q_{O_2} for the succinoxidase. Since the test for the latter involves the activity of the former, one might wonder how much of the effect of cobalt on succinoxidase is attributable to its inhibition of cytochrome oxidase.

We tested the effect of cobalt on succinic dehydrogenase activity aerobically without involving the cytochrome oxidase system by measuring the rate of reduction of triphenyltetrazolium chloride by liver homogenates in the presence of succinate (17). Under these conditions cobalt effected an inhibition of succinic dehydrogenase of 60–70%. The data thus indicate that aerobically both cytochrome oxidase and succinic dehydrogenase in homogenates are inhibited by cobaltous ions.

Of the enzymes tested, cobalt exerted significant inhibitory action on only two -SH-dependent enzymes (succinic dehydrogenase and choline oxidase) and on two -SH nondependent enzymes (cytochrome oxidase and catalase). Therefore, cobalt cannot be considered as an inhibitor of all -SH enzymes nor as specific for only -SH enzymes.

Only with choline oxidase does it appear that simple mercaptide formation might be the explanation for the observed inhibition since this enzyme alone is equally affected by cobalt under both aerobic and anaerobic conditions.

TABLE I
Effect of Cobalt on Oxidative Enzymes
A. Aerobically in Warburg respirometers
37°C.

| Enzyme | Tissue | QO ₂ | | Inhibition | -SH-dependent |
|--------------------|--------|-----------------|------------------|------------|---------------|
| | | Control | Co ⁺⁺ | | |
| Succinoxidase | Liver | 46 | 18 | % 60 | Yes |
| | Kidney | 90 | 44 | 51 | Yes |
| Cytochrome oxidase | Liver | 56 | 30 | 46 | No |
| Choline oxidase | Liver | 2.25 | 0.35 | 84 | Yes |

There was no effect by cobalt on malic oxidase (-SH-dependent), lactic oxidase (-SH-independent) or cytochrome reductase (-SH-independent).

B. Anaerobically in Thunberg tubes

| Enzyme | Inhibition | Hydrogen acceptor |
|------------------------|------------|---|
| Succinic dehydrogenase | 0-30 | Methylene blue, or triphenyl tetrazolium chloride |
| Choline dehydrogenase | 70-100 | Chlorphenol-indophenol |

There was no effect on malic or lactic dehydrogenase.

With the succinoxidase system, whose anaerobic inhibition by cobalt is weak, it is deemed likely that either (1) oxygen is involved in the formation of a stable combination between Co⁺⁺ and the dehydrogenase, in a manner analogous to the formation of complexes between cobalt, histidine, and oxygen (16), or (2) under aerobic conditions, cobalt acts on some unknown factor in the succinoxidase system.

The sensitivity of the cytochrome, succinic, and choline oxidase systems in rat liver and kidney homogenates to 1×10^{-3} M cobalt appears sufficient to account for the observed inhibition by cobalt of the respi-

TABLE II

Effect of Cobalt on Phosphorylytic Enzymes

Activities are expressed as $\mu\text{g.}$ of inorganic phosphorus liberated from the indicated substrate by 1 mg. dry tissue/indicated time. Corrections for inorganic phosphorus in the tissue and substrate have been applied.

| Enzyme | Tissue | Control | Co ⁺⁺ | Time | -SH-dependence |
|-----------------------------|--------|---------|------------------|-------------|----------------|
| | | | | <i>min.</i> | |
| Aerobic | | | | | |
| Adenosinetriphosphatase | L | 44 | 48 | 15 | Yes |
| | K | 48 | 42 | 15 | |
| Anaerobic | | | | | |
| Adenosinetriphosphatase | L | 46 | 44 | 15 | |
| | K | 50 | 48 | 15 | |
| β -Glycerophosphatase | L | 8 | 8 | 60 | No |
| | K | 12 | 12 | 60 | No |
| Nucleotidase | K | 4.4 | 4.0 | | ? |

TABLE III

The Effect of Cobalt on Catalase Activity in Liver
(-SH-independent)

The data are expressed as k , the reaction-rate constant for a first-order reaction. ($k = 2.303/t \log a/(a - x)$, where t = time in minutes, a = original concentration of peroxide and $a - x$ = concentration of peroxide remaining at time t .)

$t = 6^\circ\text{C.}$

| Time | 3 | 6 | 9 | 12 | 15 |
|------------------------------|--------|--------|--------|--------|--------|
| <i>min.</i> | | | | | |
| Control | 0.1015 | 0.1003 | 0.0953 | 0.0940 | 0.0930 |
| Cobalt, $1 \times 10^{-3} M$ | 0.0660 | 0.0610 | 0.0535 | 0.0520 | 0.0490 |
| Inhibition, % | 35 | 39 | 44 | 45 | 47 |

ration of intact slices of these tissues. The inhibition of catalase may also be involved in the progressive decrease of respiration of the slices with time of contact of cobalt with the tissue.

SUMMARY

Ten enzymes or enzyme systems, both -SH-dependent and -SH-independent, found in homogenates of rat liver and kidney were tested for sensitivity to $1 \times 10^{-3} M$ cobaltous ions. Succinoxidase, choline

oxidase, adenosinetriphosphatase, malic oxidase, and lactic oxidase were studied under both aerobic and anaerobic conditions while cytochrome oxidase, catalase, cytochrome reductase, β -glycerophosphatase, and nucleotidase were tested aerobically.

Under aerobic conditions, cobalt had a marked inhibitory action on only two of the four -SH-dependent enzyme systems: succinoxidase and choline oxidase, and on two of the five -SH independent enzyme systems: cytochrome oxidase and catalase. Of those systems tested anaerobically, only choline dehydrogenase and succinic dehydrogenase were inhibited by cobalt although the latter enzyme was much less sensitive under these conditions than under aerobic conditions.

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Cytochrome Oxidase in the Potato Tuber¹

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INTRODUCTION

This paper will present evidence confirming the conclusion of Levy and Schade (8) that cytochrome oxidase occurs in the tuber of potato (*Solanum tuberosum*). The potato tuber has long been used as a source of tyrosinase (polyphenol oxidase) and Kubowitz (6,7) has isolated this oxidase from potato and demonstrated that it is a copper protein of high activity. He showed that the oxidase could be used to oxidize catalytically glucose-6-phosphate to phosphogluconic acid by molecular oxygen, in the presence of catalytic quantities of catechol, triphosphopyridine nucleotide, and glucose-6-phosphate dehydrogenase. Further, Boswell and Whiting (2,3) have concluded from their experiments that tyrosinase is the terminal oxidase in this tissue, and this view was substantiated by the experiments of Baker and Nelson (1) who showed that a major fraction of the respiration was inhibited by 2,4-dinitrocatechol, a presumed competitive inhibitor of tyrosinase.

Schade, Bergmann, and Byer (9) have shown that the oxidized products of catechol are cellular poisons, and that the use of catechol to identify tyrosinase as the terminal oxidase is of dubious value. This conclusion, of itself, need not invalidate the functional role of tyrosinase since other substituted dihydroxyphenols, such as caffeic acid and dihydroxyphenylalanine, may be the functional carriers. Whether one accepts Levy and Schade's conclusion that tyrosinase probably plays no functional role in potato tuber respiration, the results reported here establish, in agreement with Levy and Schade, the presence of both tyrosinase and cytochrome oxidase activity in the potato. Further, the experiments reported here make it clear that the cytochrome oxidase

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activity is due to a distinct enzyme, and is not due to a residual activity of tyrosinase and contaminating polyphenols accepting electrons from cytochrome c. Though it is of interest that potato, long considered a tyrosinase (polyphenol oxidase) plant, contains an active cytochrome oxidase, no conclusion can be drawn from these experiments as to the relative roles of tyrosinase and cytochrome oxidase in the respiration of the intact tissue.

EXPERIMENTAL

Materials and Methods

White potatoes of unknown variety were obtained on the market as needed; they were washed and peeled. The cortex (tissue outside the vascular ring) was removed, and 100 g. of the cortex and peelings were blended for 5 min. with 100 ml. of 0.05 *M* sodium barbiturate. This was then filtered through cheesecloth and centrifuged for 15–20 min. at $500 \times g$ and the sediment discarded. The supernatant liquid was assayed directly for cytochrome oxidase and tyrosinase, or was dialyzed in the cold for 24–48 hr. against 0.01 *M* sodium barbiturate, without appreciable loss of activity of either enzyme. The undialyzed supernatants will be referred to as the crude preparations.

The cytochrome oxidase activity was followed in the Warburg respirometer by the increase in oxygen uptake with the addition of cytochrome c, over that without cytochrome. The electron donor used was 3.0 mg. of hydroquinone in 0.1 ml. added to the vessel at zero time from the side arms. The temperature was 25°C., and the buffer was potassium phosphate, 0.1 *M*, pH 7.1.

The tyrosinase activity was similarly tested using 1.0 ml. of enzyme diluted 1:10, at pH 6.8, and with 0.1 mg. of catechol in place of cytochrome c as mediator of the hydroquinone oxidation. With this test linear curves of oxygen uptake with time are obtained until all of the hydroquinone is oxidized. If catechol is used as an electron donor (3.0 mg. catechol), higher initial rates of oxygen uptake are observed with rapid inactivation of the enzyme.

RESULTS

Homogenates and crude preparations contain both cytochrome oxidase and tyrosinase as may be seen from the results reported in Table I. The cytochrome oxidase activity is only about 3% that of the tyrosinase activity, but it is still adequate to account for all the respiration. The cytochrome oxidase activity of the extracts, calculated on the basis of the original amount of tissue, equals 180 μ l. O_2 /g. wet wt. tissue/hr. while the rate of respiration of tissue slices is 35–75 μ l. O_2 /g. wet wt. of tissue/hr. (2,3).

The specificity of the cytochrome and catechol oxidations make it extremely probable that two distinct enzymes are contained in the crude preparation. There is, however, the possibility that cytochrome c

TABLE I

*Cytochrome Oxidase and Tyrosinase Activity*Microliters O₂ in 50 min.; substrate 3.0 mg. hydroquinone; temp. 25°C.

| Oxidase | Cytochrome oxidase pH 7.1; 1.0 ml. oxidase | | Tyrosinase pH 6.8; 0.1 ml. enzyme | | Ratio: tyro- sinase: cyto- chrome oxi- dase |
|--|---|----------------------|--------------------------------------|-------------|--|
| | cytochrome c 4.5 × 10 ⁻⁶ M | no cyto- chrome c | catechol 0.1 mg. | no catechol | |
| Prepn. A; homogenate | 123 | 14 | 333 | 1 | 31 |
| Prepn. B; supernatant, 500 × g for 10 min. | 104 | 19 | 337 | 2 | 39 |
| Prepn. C; supernatant from 16,000 × g for 15 min. | 19 | 15 | 260 | 2 | 616 |
| Prepn. D; sediment from 16,000 × g for 15 min. Per cent in sediment Recovery (C + D) in per cent of B | 53 (60) (63) | 3 | 38 (11) (88) | 0 | 7.6 |
| Prepn. F; sediment from 16,000 × g washed twice at 16,000 × g, made up to ½ vol. Recovery, per cent of B | 167 (47.5) | 5 | 76 (5.6) | 0 | 4.6 |

undergoes oxidation at the expense of traces of quinones formed by tyrosinase. The experiments reported below should eliminate this possibility.

Though we have not succeeded in preparing from potatoes a cytochrome oxidase free of tyrosinase, we have been able, by high speed centrifugation, to increase the relative activity of cytochrome oxidase to tyrosinase. We may represent the relative activity as a ratio defined as follows:

$$\text{Ratio} = \frac{\mu\text{l. O}_2 \text{ in 50 min./ml. extract with catechol}}{\mu\text{l. O}_2 \text{ in 50 min./ml. extract with cytochrome c}}$$

Where catechol and cytochrome c, respectively, function catalytically in the transfer of electrons from hydroquinone; the values used in

calculating the ratio are determined after correction for blanks run without cytochrome *c* or catechol. Results are shown in Table I. The crude preparation, *B*, has a ratio of 39. Most of the tyrosinase of these preparations is in "solution" as is shown by comparing the activities of *C* and *D*, while most of the cytochrome oxidase activity is in the sediment. After washing the sediment twice at $16,000 \times g$, the ratio has fallen from the initial value of 39 to 4.6. The tyrosinase activity found in the sediment of *F* may be due only to contamination, or it may be bound in part to the same particles that contain the cytochrome oxidase. The low recovery of 47.5% may be due mostly to considerable cytochrome oxidase destruction during centrifugation, and in part to the fact that some of the activity remains in the supernatant. In one experiment at $18,000 \times g$ for 100 min., 82% of the residual activity was found in the sediment, 18% in the supernatant. No appreciable loss of tyrosinase occurs during the high speed centrifugation.

TABLE II

The Inhibition of Tyrosinase by Phenylthiourea

Substrate, hydroquinone, 6.0 mg.; $t = 25^\circ\text{C}$.; pH 7.1; phenylthiourea added as solid crystals; enzyme dialyzed 48 hr. against 0.01 *M* sodium barbiturate.

| Oxidase, ml. | 1.0 | 1.0 | 1.0 | 0.5 | 0.5 |
|--|------------------------|------------------------|------|------|-------|
| Cytochrome <i>c</i> | $4.5 \times 10^{-5} M$ | $4.5 \times 10^{-5} M$ | none | none | none |
| Catechol, mg. | none | none | none | 0.1 | 0.1 |
| Phenylthiourea | none | satd. | none | none | satd. |
| Expt. A, $\mu\text{l. O}_2$ in 45 min. | 69 | 46 | 7 | 592 | 12 |
| Inhibition, % | | 33 | | | 98 |
| Expt. B, $\mu\text{l. O}_2$ in 45 min. | 78 | 48 | — | — | — |
| Inhibition, % | | 38 | | | |

Phenylthiourea is a powerful inhibitor of tyrosinase (4) but inhibits cytochrome oxidase to a much smaller extent, as is shown by the results reported in Table II. It will be seen that in the presence of phenylthiourea the cytochrome-catalyzed oxidation is much larger than that in the presence of catechol, so that one cannot account for cytochrome oxidase activity as a residual tyrosinase activity.

Keilin (5) reported that the inhibition of tyrosinase by carbon monoxide (CO), unlike the CO inhibition of cytochrome oxidase, is not reversed by light. The results reported in Table III show that the

tyrosinase activity is CO-sensitive and not light-reversible, while the cytochrome activity is photo-reversibly inhibited by CO. The differential action of light on the CO-inhibition of the two catalytic activities of the crude oxidase is strong evidence for the presence of two distinct enzymes.

TABLE III

Carbon-Monoxide Inhibition of Cytochrome Oxidase and Tyrosinase
 Microliters O₂/60 min.; hydroquinone 3.0 mg.; temp. 25°C.; pH 7.1

| Gas phase | Cytochrome oxidase 1.0 ml. oxidase; cytochrome c, 4.5×10^{-5} M | | Tyrosinase 1.0 ml. oxidase (diluted 1:5); catechol, 0.1 mg. | |
|---------------------------------------|---|------|--|------|
| | Light | Dark | Light | Dark |
| 95% CO/5% O ₂ | 117 | 29 | 78 | 64 |
| 95% N ₂ /5% O ₂ | 154 | 149 | 264 | 272 |

Some crude preparations are active in the oxidation of hydroquinone without the addition of catechol or cytochrome c, due presumably to polyphenols present, since this effect can be largely abolished by dialysis. For example, in one experiment undialyzed crude oxidase with hydroquinone at 25°C. and pH 6.8 gave an O₂ uptake of 34.8 μ l. O₂/ml. of enzyme in 30 min.; after 24 hr. dialysis this had decreased to 5.1 μ l. The increase in O₂-uptake upon the addition of cytochrome c was 32.3 μ l. O₂ in the undialyzed preparation and 32.5 μ l. O₂ for the dialyzed preparation. The oxidase used in the experiments reported in Table II was well dialyzed, and had retained most of its initial activity. It seems highly improbable that the oxidation of cytochrome c in the dialyzed extracts is due to oxidation of the cytochrome by a quinone produced through the action of tyrosinase.

Preparations of tyrosinase can be made which are free of cytochrome oxidase, as shown either in the respirometer or by much more sensitive spectrophotometric tests. Tyrosinase preparations free of cytochrome

TABLE IV

Tyrosinase Activity Free of Cytochrome Oxidase

Acetone precipitated oxidase; hydroquinone 6.0 mg., $t = 25^\circ\text{C}.$; buffer 0.1 M phosphate, pH 7.1.

| | | | | |
|------------------------------------|-----|--------------|----------------------|-----|
| Oxidase, ml. | 0.1 | 0.1 (boiled) | 1.0 | 1.0 |
| Catechol, mg. | 0.1 | 0.1 | — | — |
| Cytochrome c | — | — | 4×10^{-5} M | — |
| μ l. O ₂ in 35 min. | 497 | 0.0 | — | — |
| μ l. O ₂ in 65 min. | — | — | 9.4 | 5.8 |

oxidase were prepared as follows: Outside bark and peelings were dehydrated by blending with 4 ml. of acetone for each gram of tissue, filtered on a Büchner funnel, washed with acetone and ether, and air-dried. To 25 g. of dry powder, 100 ml. of ice-cold distilled water was added, blended for 5 min., and centrifuged at $1200 \times g$ for 20 min. To 60 ml. of the supernatant, 40 ml. of ice-cold acetone was added, and packed in ice until a slight precipitate was formed. The precipitate was removed by centrifugation and discarded. To the supernatant was added 15 ml. of cold acetone, the precipitate was collected on the centrifuge, and the supernatant was discarded. The precipitate, which was nearly free of color, was dissolved in 4 ml. of water and dialyzed in the cold for 24 hr. against 0.01 *M* sodium barbiturate.

The purified tyrosinase preparation was tested for cytochrome oxidase and tyrosinase activities as described above. Essentially no cytochrome oxidase activity was detected in the respirometric test reported in Table IV, even though the preparation was extremely active in the catechol test.

TABLE V

Oxidation of Ferrocyanochrome c Followed Spectrophotometrically at 550 m μ

Three ml. of 3.0×10^{-5} *M* cytochrome c in 0.05 *M* phosphate buffer, pH 7.1; temperature 25°C.

Optical density ($\log I_0/I$)

| Time | Water, 0.2 ml. | Tyrosinase, ^a 0.2 ml. | Water, 0.5 ml. | Cytochrome oxidase ^b diluted 1:10, 0.5 ml. | Cytochrome oxidase ^b diluted 1:50, 0.5 ml. |
|-------------|-------------------|-------------------------------------|-------------------|--|--|
| <i>min.</i> | | | | | |
| 0.0 | .79 | .86 ^c | .65 | >.79 ^c | .76 |
| 0.5 | | | | .62 | |
| 1.0 | | .87 | .67 | | |
| 1.5 | | | | .48 | |
| 2.5 | .76 | | | .39 | .62 |
| 3.5 | | .84 | | .33 | .60 |
| 4.5 | | .84 | | | .58 |
| 6.5 | | | .66 | | .56 |
| 10.0 | .71 | .82 | | | |
| 11.5 | | | | | .47 |
| 15.0 | .72 | .81 | | | |
| 20.0 | .66 | .79 | .64 | | .42 |

^a With hydroquinone and 0.1 mg. catechol, 0.1 ml. of this preparation consumed 396 μ l. O₂ in 30 min.

^b Enzyme dialyzed 48 hr.

^c Some initial increase, because enzyme preparations not optically clear.

A far more sensitive test for cytochrome oxidase is obtained by following its activity in the oxidation of cytochrome c by the change in light absorption in the spectrophotometer. The cytochrome c was reduced by 0.001 *M* sodium dithionite in 0.1 *M* phosphate buffer at pH 7.1; the excess dithionite was removed by aeration. The final concentration of cytochrome in the absorption cells was 3×10^{-5} *M*, and since the molar concentration of O_2 in water in equilibrium with air is 2.6×10^{-4} *M*, a great excess of O_2 is present. After reading the optical density of the ferrocytochrome c at 550 $m\mu$, water or dilute enzyme is added at a temperature of 0°C., and the change in optical density at 550 $m\mu$ is followed at successive times on the Beckman spectrophotometer. There is an initial fall in optical density when water is added, for which correction can be made, and a slight initial rise on the addition of the oxidase, since the oxidase is not optically clear. With dialyzed oxidase, diluted 1:50 with water, 0.5 ml. of oxidase in a total volume of 3.5 ml., a rapid oxidation of ferrocytochrome c to ferricytochrome c occurs as may be seen in Table V. When the tyrosinase which has been purified by acetone treatment is used, no greater oxidation occurs than is found upon the addition of water. This experiment clearly demonstrates that a very powerful tyrosinase from potatoes is without action in the catalytic oxidation of cytochrome c, and that the cytochrome c activity of the crude oxidase must be due to an additional component, cytochrome oxidase.

SUMMARY

Though a very active tyrosinase occurs in the potato, we also find, in agreement with the conclusions of Levy and Schade, a cytochrome oxidase which is sufficiently active to account for the total respiration of the tissue. The cytochrome oxidase activity is due to a distinct enzyme, and not to residual activity of tyrosinase, nor does it depend upon the presence of polyphenols. The cytochrome oxidase activity is but little inhibited by phenylthiourea, while this inhibitor abolishes the activity of tyrosinase. The cytochrome oxidase is photo-reversibly inhibited by carbon monoxide, while the catechol-oxidase inhibition by carbon monoxide is not light-reversible.

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Age of Animals in Relation to the Utilization of Calcium and Magnesium in the Presence of Oxalates¹

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INTRODUCTION

The concept that oxalates in foods interfere with the assimilation of calcium is widely accepted. This opinion concerning the unutilizable nature of calcium oxalate is based upon determinations of the calcium content of the bodies of young rats fed upon diets containing oxalate or some foodstuff such as spinach (1). The experiments in this field were usually terminated when rats were about 60 days old. A number of studies (2, 3) indicate that children and rats can store calcium without serious interference by oxalate-containing foods.

The potential wastage of calcium by oxalates contained in foods has assumed additional interest due to the observations that foods rich in oxalate, such as rhubarb, will protect teeth against acid erosion from cola beverages and lemon juice (4). A beverage composed of equal volumes of lemon juice or lemonade and rhubarb juice will not attack the enamel of teeth. This protection is important to the people in the older age groups who take large amounts of lemon juice in the hopes of alleviating constipation or arthritis. This same age group is especially liable to be in negative calcium balance (5). Therefore, it can ill afford wastage of calcium from combination with oxalate.

The relative assimilability of different salts of calcium in relation to the age of an animal has been given little attention since it is commonly assumed that studies made with young, growing animals apply to all stages of life. This becomes of practical significance in the case of such common foodstuffs as bread. Part of the calcium in bread originates

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from milk; another substantial fraction comes from the yeast foods in the form of calcium sulfate. If different forms of calcium are absorbed to different degrees between youth and old age, this knowledge becomes important since the diets of the aged include large amounts of bread.

The present studies were designed first to determine if the degree of assimilation of calcium in the presence of oxalate varied in relation to the age of the animal and secondly to learn if consuming calcium and oxalate at two different meals might increase the absorption of the calcium.

EXPERIMENTAL

The diet consisted of 4 parts: (a) a basal "B" diet consisting of a mixture of toasted corn flakes, 42 g.; brewers' yeast, 5 g.; and cod liver oil, 3 g.; (b) 10% aqueous sucrose solution; (c) evaporated milk; and (d) steamed ground horse meat containing a mixture of muscle and liver.

The composition of these dietary components is given in Table I. About $\frac{1}{3}$ of the calcium came from the cereal part of the diet and the remainder from milk.

TABLE I
Composition of Diet Components

| Material | Water | Dry matter | Ash | Ca ^a | Mg ^a | P ^a |
|------------|-------|------------|-----|-----------------|-----------------|----------------|
| | % | % | % | mg./g. | mg./g. | mg./g. |
| Diet "B" | 7.3 | 92.7 | 3.8 | 1.5 | 2.5 | 6.0 |
| Evap. milk | 74.8 | 25.2 | 6.2 | 10.5 | 1.5 | 7.1 |
| Meat | 67.1 | 33.9 | 3.1 | 0.3 | 0.6 | 5.1 |

^a Dry basis.

Shortly after weaning, 18 young rats were divided into 3 groups using litter mate and sex distribution. Balances were run upon each rat for calcium and magnesium during 12 periods of 10 days each. In each case collections were made for 7 days after a preliminary period of feeding the diet for 3 or 4 days. These balances were continuous starting when the rats were 38 days old. The mean age of the rats was 175 days at the conclusion of the 12 periods mentioned below.

The feeding regime outlined below was followed:

Period I

Group I. A. m.: meat, 5 g., plus 1 ml. of 10% sucrose solution; p.m.: Diet "B," 2.5 g., evaporated milk, 5 g.; and potassium oxalate, 60 mg.; dissolved in 1 ml. of 10% aqueous sucrose solution.

Group II. Same as Group I except the oxalate was fed with the meat in the morning, thus allowing the oxalate to be in the stomach 5 hr. before the milk.

Group III. Same as Group I except the milk and diet "B" were fed in the morning and the meat with oxalate was fed 5 hr. later.

Period II

The same regime as in Period I with oxalate omitted entirely to permit the rats to remedy body depletion of calcium.

Period III

Group I. The same as for Period I.

Group II. Treated as Group III in Period I.

Group III. Treated as Group II in Period I.

The remainder of the twelve periods represented a repetition of this cycle for the first three with the food allowances increased as the rats grew.

The amount of oxalate was adjusted to combine with 75% of all the calcium in the diet.

From the time the rats were 175 days old until they were 438 they were maintained upon a stock diet containing 3.3% calcium.

Beginning at the mean age of 438 days, two 14-day periods were run. A 10-day collection followed a 4-day preliminary feeding period. In the first of these two periods all rats were fed according to the regime of Group I in Period I with calcium and oxalate together. In the second of these two periods the regime of Group I in Period II was followed with no oxalate in the diet.

The feeding regime allowed all the growing rats one period of 10 days out of each 30 days to build body stores of calcium without interference from oxalate. During the other two periods, Group I consumed a diet in which three-fourths of the calcium was subject to reaction with oxalate. Groups II and III were fed the same amounts of oxalate and calcium, except that periods of 5 hr. intervened between the feeding of oxalate and calcium, so that one or the other ion would have a start in moving through the gastrointestinal tract.

As the rats grew, the weekly feed ingested on a dry basis increased from 43 g. in Period I to 61 g. in Period III. The ingested values for calcium, magnesium, and phosphorus, expressed in milligrams per week, were 128-193, 78-97, and 205-354, respectively. Thus, on a dry basis, the diet provided 0.3% calcium which is well recognized as a marginal rather than an optimal allowance. This level was used since it corresponds to that found in the diets of older people.

Accepted analytical methods described in A. O. A. C. Methods were employed.

Only detailed data are presented for calcium since such values indicating individual variations during the growing period are not available elsewhere (Table II). The data upon magnesium according to regime are summarized in Table III.

RESULTS

The summaries of data (Tables II and III) indicate that the average young rat during the first 2 months of life can make little use of the calcium of milk that is subject to combination with oxalate. During Periods I and III, when the oxalate was fed with the milk, the rat absorbed about one-fourth of the dietary calcium. Without any oxalate in the diet, these same rats (Period II) retained 94% of the calcium.

Separating the feeding of oxalate and calcium by a time interval of 5 hr. nearly doubled the retention of calcium until the rats were

TABLE II
*Range in Percentage of Calcium Retained by
Individual Rats with Increasing Age*

| Group | Period | Mean body weight | Age range (days) | Percentage retained | | Regime |
|-------|--------|------------------------|------------------------|---------------------|---------|---|
| | | | | Mean | Range | |
| I | 1 | 87 | 38-51 | +27 | -10-+59 | A.m.: meat; p.m.: milk and oxalate. |
| | 3 | 121 | 59-72 | +30 | +15-+44 | |
| | 4 | 135 | 70-83 | +43 | +31-+55 | |
| | 6 | 166 | 91-104 | +79 | +73-+85 | |
| | 7 | 179 | 101-114 | +77 | +64-+88 | |
| | 9 | 193 | 123-136 | +69 | +59-+77 | |
| | 10 | 224 | 143-156 | +42 | +10-+62 | |
| | 12 | 244 | 164-177 | +44 | +22-+74 | |
| | 13 | 296 | 425-431 | -10 | -18-- 3 | |
| | 2 | 105 | 49-62 | +91 | +89-+94 | Same regime but no oxalate. |
| | 5 | 149 | 80-93 | +92 | +91-+93 | |
| | 8 | 186 | 112-125 | +88 | +86-+91 | |
| | 11 | 235 | 154-167 | +59 | +49-+76 | |
| | 14 | 302 | 439-445 | + 6 | - 3-+16 | |
| II | 1 | 86 | 38-51 | +41 | + 9-+73 | In periods 1, 4, 7, and 10, a.m.: meat and oxalate; p.m.: milk. In periods 3, 6, 9, and 12, a.m.: milk; p.m.: meat and oxalate. |
| | 3 | 119 | 59-72 | +51 | +13-+81 | |
| | 4 | 134 | 70-83 | +72 | +38-+87 | |
| | 6 | 167 | 91-104 | +81 | +76-+83 | |
| | 7 | 178 | 101-114 | +79 | +69-+85 | |
| | 9 | 189 | 123-136 | +73 | +53-+96 | |
| | 10 | 223 | 143-156 | +57 | +32-+79 | |
| | 12 | 240 | 164-177 | +47 | +26-+63 | |
| | 13 | 272 | 425-431 | -17 | -34-- 3 | |
| | | | | | | Same as Gr. I, Per. I. |

TABLE II—*Continued*

| Group | Period | Mean body weight | Age range (days) | Percentage retained | | Regime |
|-------|--------|------------------------|------------------------|---------------------|---------|--|
| | | | | Mean | Range | |
| | 2 | 104 | 49-62 | +91 | +87-+94 | Same regime as in periods 1, 4, 7, 10, except no oxalate. |
| | 5 | 149 | 80-93 | +91 | +89-+93 | |
| | 8 | 186 | 112-125 | +83 | +71-+91 | |
| | 11 | 235 | 154-167 | +62 | +50-+73 | |
| | 14 | 258 | 439-445 | + 1 | - 7-+ 9 | Same as Gr. I, Per. II. |
| III | 1 | 83 | 38-51 | +48 | + 8-+61 | In periods 1, 4, 7, 10, a.m.: milk; p.m.: meat and oxalate. In periods 3, 6, 9, 12, a.m.: meat and oxalate; p.m.: milk. |
| | 3 | 116 | 59-72 | +58 | +33-+76 | |
| | 4 | 124 | 70-83 | +74 | +50-+83 | |
| | 6 | 160 | 91-104 | +80 | +74-+88 | |
| | 7 | 170 | 101-114 | +77 | +62-+88 | |
| | 9 | 185 | 123-136 | +70 | +64-+80 | |
| | 10 | 211 | 143-156 | +50 | +27-+71 | |
| | 12 | 230 | 164-177 | +50 | +40-+62 | |
| | 13 | 309 | 425-431 | -16 | -39--.5 | Same as Gr. I, Per. I. |
| | 2 | 100 | 49-62 | +90 | +83-+93 | Same regime as in periods 1, 4, 7, 10, except no oxalate. |
| | 5 | 139 | 80-93 | +88 | +82-+93 | |
| | 8 | 176 | 112-125 | +82 | +69-+88 | |
| | 11 | 222 | 154-167 | +66 | +46-+81 | |
| | 14 | 287 | 439-445 | + 6 | + 2-+ 8 | Same as Gr. I, Per. II. |

about 12 weeks old. Without oxalate in the diet, these rats retained 88-91% of the calcium.

As the rats grew older, the percentage of retained calcium increased until a maximum was reached at a mean age of 98-108 days. At these ages the rats retained 77-86% of all the calcium in the diet in spite of the ingestion of sufficient oxalate to bind 75% of this calcium. At the same time the spacing had little effect since the utilization of calcium was nearly as good as it was when the diet contained no oxalate.

The decrease in the need for calcium after the skeleton is well developed appeared at the ninth period when the rats were 130 days old. When the rats were 160-170 days old, they retained 44-50% of the calcium if the diets contained oxalate and 60-66% without it. Finally, when the rats attained an age of 450 days, their calcium retention was about 10% without oxalate and they were in negative calcium balance when oxalate was fed.

The individual variability (Table II) is marked if the diet contains oxalate during the period immediately after weaning. Under the conditions of this study with the diet providing enough oxalate to bind three-fourths of the calcium, some individuals could store only 10% or less of the available calcium if oxalate were present. This would amount to less than half of the unbound calcium. This would seem to indicate that during these early weeks after weaning, oxalate may exert some effect on calcium absorption beyond its capacity to produce

TABLE III
Percentage Retention of Magnesium by Rats at Different Ages

| Period | Mean wt. | Mean age | Oxalate with Ca | Oxalate before Ca | Oxalate after Ca |
|----------------|-----------|-------------|-----------------|-------------------|------------------|
| | <i>g.</i> | <i>days</i> | | | |
| 1 | 87 | 44 | 36 | 37 | 24 |
| 3 | 121 | 66 | 23 | 35 | 46 |
| 4 | 135 | 76 | 37 | 37 | 33 |
| 6 | 166 | 98 | 32 | 40 | 38 |
| 7 | 179 | 108 | 40 | 36 | 37 |
| 9 | 193 | 130 | 40 | 34 | 39 |
| 10 | 224 | 149 | 19 | 27 | 26 |
| 12 | 240 | 170 | 22 | 22 | 25 |
| 13 | 296 | 428 | 7 | 5 | 2 |
| No oxalate fed | | | | | |
| 2 | 105 | 56 | 44 | 41 | 49 |
| 5 | 149 | 86 | 35 | 39 | 38 |
| 8 | 186 | 118 | 35 | 40 | 39 |
| 11 | 235 | 160 | 22 | 19 | 29 |
| 14 | 302 | 442 | 15 | 14 | 14 |

an insoluble salt. Even during the first period, however, when the rats were 38–51 days old, there were individuals that could store a third of the calcium bound by oxalate as well as an equal amount that was free. Spacing by a time interval increased this ability of favored individuals so that a few could store three-fourths of the dietary calcium even during the first periods (Fig. 1).

These results may point to one of the problems in the case of many young animals that pass through a period of poor health in making the transition from the mother's milk to solid foods. The form of calcium

may be very important during this period. Without the oxalate during the early periods, the mean assimilation was excellent as indicated by the three percentages for mean storage of 91, 91, and 90. Variability between extremes for individuals was only 10% when the diet contained no insoluble form of calcium and most of the calcium was derived from milk.

The decline in calcium assimilation as the animal passes through middle age is very marked. In the presence of oxalate the older rat tends to go into negative balance earlier than it would normally (Fig. 2). This may indicate that the form of calcium during the latter third of life is just as important as it is during the early months after weaning.

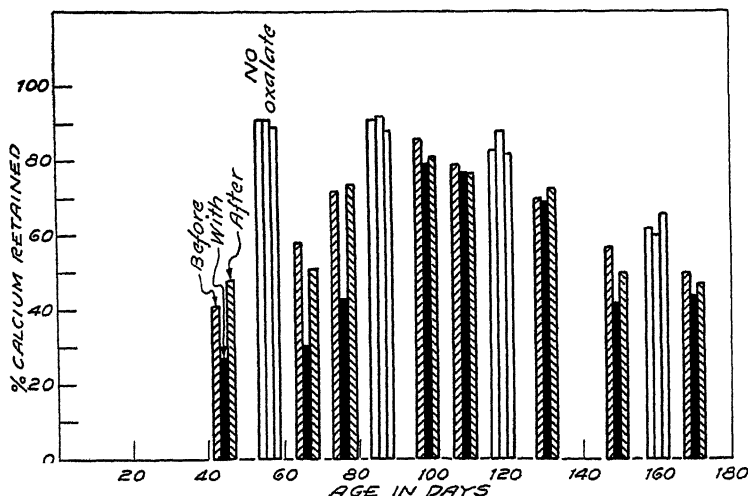


FIG. 1. Calcium absorption as affected by feeding oxalate before, with, and after the calcium.

These results may explain the discrepancy between the findings of Mackenzie and McCollum (3) and those of Fairbanks and Mitchell (1) or Mueller and Cooney (6). Mackenzie and McCollum found the ash content of the bones of rats was little affected by high levels of oxalate. Their rats were not killed until 10 weeks after weaning. Other workers ran ash determinations after the rats had been on the diets for 5 weeks. These shorter periods probably did not allow the rats to enter the age

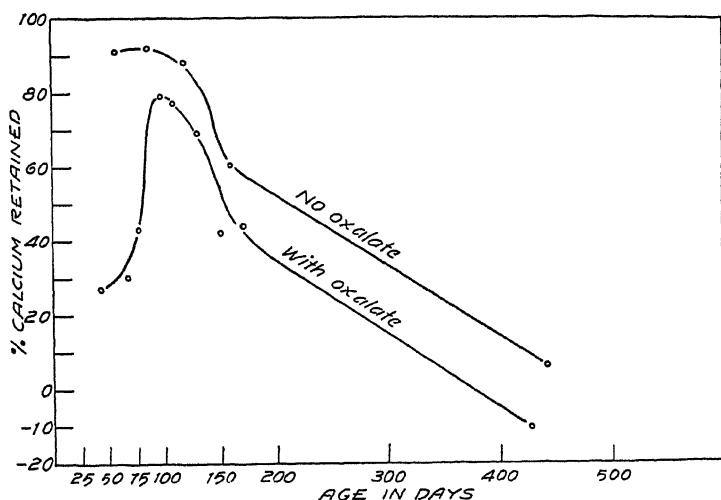


FIG. 2. Effect of dietary oxalate upon calcium absorption by rats in different periods of the life span.

period when they can assimilate calcium in the presence of oxalates. Hence, the bones of these younger rats were low in ash.

Since magnesium oxalate is rather insoluble some assumptions have been made by Wittwer (7) that oxalate in spinach might also bind this element. The results from magnesium balances (Table III) provide no evidence that oxalate in the amounts used in these studies affected the storage of magnesium.

SUMMARY

The utilization of calcium in the presence of oxalate varies depending upon the age of the rat. When enough oxalate was included in the diet to combine with three-fourths of the calcium, only one-fourth of the dietary calcium was absorbed when the rat was 50 days old, but 80% of the dietary calcium was utilized by the time the rat attained an age of 100 days. After this age, the utilization declines steadily to a low value as shown by the balance studies made at the mean age of 438 days. At this time the balance is still positive when the diet contains 0.3% calcium but it is negative when this diet contains oxalate fed with the calcium in the form of milk. Oxalate fed either 5 hr. before or 5 hr. after calcium permits more efficient utilization even in young rats shortly after the weaning age.

Magnesium absorption was not affected in these studies since the level of oxalate was low.

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Constancy of Nucleic Acid and Phospholipide Phosphorus With Aging in Mouse Liver¹

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INTRODUCTION

Nucleic acids have been linked with protein synthesis by a number of workers among whom are Caspersson (1) and Brachet (2). Observations of Caspersson and Schultz (3) indicate that intense protein production, as in active growth, is associated with an increase in nucleic acid concentration. It is reasonable to expect a decrease in protein synthesis, and therefore in nucleic acid levels, on aging.

Several investigators have reported a progressive decrease in nucleic acid concentration during embryonic development: Masing (4), Graff and Barth (5), Caspersson and Thorell (6), and Dumm (7). It was found by Davidson and Waymouth (8) and by Robertson and Dawbarn (9) that tissues from embryonic and new-born sheep, respectively, had higher nucleic acid contents than corresponding adult tissues. Andreassen (10) used nucleoprotein phosphorus to trace the growth of the lymphatic system in rats and found a decrease in nucleoprotein with increasing age. Lowry and his co-workers (11,12), however, have reported no significant change in total nucleic-acid phosphorus with age.

This is an attempt to amplify the available data on nucleic acid concentration as a function of age by the use of a standardized strain of mice and newer methods of analysis.

METHOD

The analytical method adopted for the present work was essentially that of Schmidt and Thannhauser (13) as modified by Schneider (14). In the original method phos-

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phorus estimations were made on the alkaline digest and on the acid filtrate, while inorganic phosphorus (from phosphoprotein) was determined in the latter by the method of Delory (15). In the present work the inorganic phosphorus determinations were omitted since it has been shown by both Schmidt and Thannhauser (13) and Davidson and Waymouth (16) as well as by early experiments in this investigation that the phosphoprotein phosphorus content of most tissues is negligible. The residue containing the desoxyribonucleic acid was treated with hot trichloroacetic acid which extracted it away from the protein, and the desoxyribonucleic acid was estimated directly by phosphorus determination on the filtrate.

EXPERIMENTAL

Groups of white mice ranging in age from 3 weeks to 13 months were used. There was no effort made to distinguish between the sexes, except that pregnant females were not used, and all had been fed a regular diet *ad lib*.

Liver tissue was used in all of the following experiments, the livers of from 3 to 5 individuals being pooled to give 5 or 6 g. of tissue for each experiment. In some of the sets the animals were killed by a blow on the head and the livers were perfused *in situ* with saline; they were then removed, blotted, and weighed. In other sets the animals were decapitated and allowed to bleed, the livers then being removed without perfusion.

After removal and weighing, the livers were transferred to a small Waring Blendor together with 100 ml. of cold trichloroacetic acid and blended for 20 sec. After homogenization was completed the material was transferred to a beaker in a bath of cracked ice and water, and the mixture was stirred slowly for 20 min.

Total phosphorus samples were taken in two different ways. In most of the sets the method used was to transfer two 1-ml. aliquots of the homogenate directly into two micro-Kjeldahl flasks at the end of this stirring period. This was usually very difficult due to plugging of the pipet so another method was also used in some sets. At the time that the livers were removed, a small piece was cut from each one. The pooled pieces were then weighed and transferred to Kjeldahl flasks.

Phosphorus determinations were done using a modification of the method of Allen (17). All determinations were run in duplicate. The color was read in a Klett-Summerson photoelectric colorimeter using a red filter. The readings were converted into micrograms of P/ml. from a calibration curve prepared using standard phosphate solutions. The colorimeter was repeatedly checked against a standard phosphate solution.

RESULTS

The original data are presented in Table I, the values being given in mg. P/100 g. of fresh tissue. The experiments in which perfusion was carried out are designated by a "p" after the experiment number. Those in which bleeding was effected by decapitation are similarly marked "d." Two columns of "Total P" values are given corresponding

TABLE I

Concentration of Phosphorus in Various Fractions of Mouse Liver

(Expressed as mg. P/100 g. fresh tissue.)

(Values may be converted into terms of nucleic acid by multiplying the P value $\times 10.1$ for desoxy- and 10.6 for ribo-.)

| Set no. | Age | No. mice | Total P (aliquot) | Total P (sample) | Acid-soluble P | Ribo P | Lipide P | Desoxy P | Residual P | Sum of fractions |
|---------|---------|----------|-------------------|------------------|----------------|--------|----------|----------|------------|------------------|
| 1 p | 3 wks. | 8 | — | — | 73 | 110 | 102 | 31 | — | 316 |
| 2 p | 5 wks. | 6 | 322 | — | 72 | 99 | 81 | 25 | — | 277 |
| 3 p | 5 wks. | 5 | 362 | — | 57 | 100 | 110 | 23 | — | 290 |
| 4 p | 6 wks. | 3 | — | 348 | 58 | 100 | 109 | 20 | 1 | 288 |
| 5 p | 2 mos. | 5 | 304 | — | 79 | 120 | 113 | 24 | — | 336 |
| 6 p | 2 mos. | 5 | 373 | — | 68 | 129 | 109 | 20 | — | 326 |
| 7 p | 2 mos. | 5 | 382 | — | 67 | 134 | 111 | 22 | — | 334 |
| 8 p | 2 mos. | 2 | 338 | 348 | 60 | 100 | 101 | 25 | — | 286 |
| 9 d | 2 mos. | 4 | — | 352 | 85 | 98 | 106 | 28 | 2 | 319 |
| 10 d | 2 mos. | 4 | — | 342 | 95 | 105 | 118 | 27 | 1 | 346 |
| 11 d | 2 mos. | 4 | — | 395 | 92 | 113 | 120 | 24 | 1 | 350 |
| 12 p | 3 mos. | 4 | — | 368 | 71 | 100 | 115 | 25 | — | 311 |
| 13 p | 3 mos. | 4 | — | 360 | 64 | 108 | 101 | 22 | 3 | 298 |
| 14 p | 4 mos. | 4 | 386 | — | 78 | 121 | 108 | 25 | — | 332 |
| 15 p | 4 mos. | 4 | — | — | 77 | 101 | 111 | 21 | — | 310 |
| 16 p | 4 mos. | 4 | — | 352 | 72 | 105 | 121 | 20 | — | 318 |
| 17 p | 4 mos. | 3 | 333 | 345 | 63 | 111 | 105 | 25 | — | 304 |
| 18 p | 6 mos. | 3 | 320 | — | 74 | 91 | 105 | 21 | — | 291 |
| 19 d | 10 mos. | 3 | — | 398 | 88 | 98 | 113 | 27 | 1 | 327 |
| 20 d | 10 mos. | 3 | — | 440 | 97 | 101 | 119 | 25 | 0 | 342 |
| 21 p | 12 mos. | 3 | — | 412 | 89 | 112 | 116 | 22 | 1 | 340 |
| 22 p | 13 mos. | 3 | 335 | — | 81 | 110 | 108 | 21 | — | 310 |
| 23 p | 13 mos. | 3 | 343 | — | 57 | 92 | 111 | 22 | — | 282 |
| 24 p | 13 mos. | 2 | 290 | — | 69 | 101 | 101 | 24 | — | 295 |
| 25 p | 13 mos. | 3 | — | 360 | 73 | 105 | 112 | 26 | 3 | 319 |
| 26 - | 6 wks. | 5 | 446 | 440 | 101 | 132 | 142 | 28 | — | 403 |
| 27 - | 11 mos. | 3 | 400 | 421 | 93 | 122 | 123 | 29 | — | 367 |

to the two methods used for obtaining specimens. In some cases the protein residual left after all the extractions had been made was analyzed for P. These values are listed under "Residual P." "Sum of Fractions" was obtained by adding acid-soluble, ribonucleic-acid, lipide, desoxyribonucleic-acid, and residual phosphorus values. Sets 26 and 27 are listed separately because in these two experiments the animals were not decapitated nor were the livers perfused with the result that the livers contained more blood than usual, giving, therefore, higher P values. Since these values are not comparable to the others these two sets were not averaged in with the rest.

TABLE II

*Concentration of Phosphorus in Various Fractions of Mouse Liver,
Expressed as Mean Values According to Age Groups*

| Age group | Acid-soluble P | Ribo-P | Lipide P | Desoxy-P | Ratio D | Total P | Sum of fractions |
|-----------------------------|----------------|--------|----------|----------|---------|---------|------------------|
| 0-2 months | 73.4 | 109.8 | 107.3 | 24.4 | 4.5 | 360.9 | 314.8 |
| Standard dev. | 13.2 | 12.8 | 10.5 | 3.3 | | 36.4 | 25.8 |
| Standard error | 3.9 | 3.9 | 3.2 | 1.0 | | 11.5 | 7.8 |
| 3-6 months | 71.4 | 105.3 | 109.5 | 22.8 | 4.6 | 354.1 | 308.7 |
| Standard dev. | — | — | — | — | | — | 13.9 |
| Standard error | — | — | — | — | | — | 5.3 |
| 10-13 months | 79.2 | 101.3 | 111.4 | 23.9 | 4.3 | 368.3 | 315.6 |
| Standard dev. ^a | 13.7 | 6.2 | 5.8 | 2.3 | | 51.5 | 22.1 |
| Standard error ^a | 5.2 | 2.3 | 2.1 | 0.86 | | 19.5 | 8.4 |

^a Values found for a standard phosphate solution showed a standard deviation of 1.22 and a standard error of 0.27, on basis of 21 determinations made at different times.

Mice up to the age of 2 months were considered "young" and those 10-13 months were called "old," leaving an intermediate group of ages 3-6 months. The mean values of these three groups together with the standard deviations and standard errors are shown in Table II. The standard deviation and standard error of a number of determinations on a known phosphate solution are also given in Table II (the mean value being 97.9).

DISCUSSION

The mean values for the various fractions in the "young" (0-2 months) and the "old" (10-13 months) groups have been compared and it has been found that none of the differences are statistically significant. However, ribonucleic-acid phosphorus (ribo P) tends to decrease with advancing age, lipide phosphorus tends to increase, while desoxyribonucleic-acid phosphorus (desoxy P) remains quite constant. The decrease seen in the total and sum-of-fractions values of the intermediate group is due to the shift in acid-soluble values largely; again a change which is not significant.

In order to interpret these results the method by which they were obtained should be critically evaluated. Schneider (14,18) has discussed the various analytical methods available and has come to the conclusion that his method (19) and that of Schmidt and Thannhauser (13) are the only ones which have been tested adequately enough to be reliable. In the present work it was attempted to check the phosphorus determinations with parallel pentose estimations early in the course of the investigation. The orcinol reaction as described by Kerr and Seraidarian (20) was used for ribonucleic acid but results were unreliable because of cloudy solutions. Very excellent checks were obtained between the desoxyribonucleic phosphorus values and those gotten with the diphenylamine test. Recently there has been much discussion as to the specificity of these pentose color reactions (13,21). Egsgaard (22) has investigated factors influencing the development of the color in the Amidol method of phosphorus determination and finds the pH and temperature to be the most important. These conditions were not controlled in this study.

There seem to be no data for mouse liver available in the literature. On comparing the data in this study with the values reported for rat liver, obtained by the same method, one finds rather good agreement, however. See Table III.

Since these data are for rats it would be interesting to compare rat and mouse values. Apparently the only data for both species reported by the same workers are two sets of total nucleic-acid phosphorus values:

| | Rat | Mouse |
|--------------------------------|---------------|-------------------|
| Davidson and Weymouth (24) | 152 (wet wt.) | 155 (wet wt.) |
| Dickens and Weil-Malherbe (25) | 310 (dry wt.) | 280-520 (dry wt.) |

It seems permissible then to compare the two species. It is to be noted that in the values given by Davidson (Table III), the nucleic-acid phosphorus is lower in the larger (older) rats.

On comparing the total phosphorus values in Table I with the sum-of-the-fractions, one is struck by the discrepancy between the two. The completeness of each extraction was checked by re-extracting the residue. Transfer of the material from the Kjeldahl flasks to test tubes after perchlorate digestion was found to be 99% complete. Finally, as shown in Table I, analysis of the residual protein at the end of the experiment revealed only negligible amounts of P to be left. This apparent loss of phosphorus then remains unexplained. McCarter and Steljes (26) carried out very careful material-balance studies using a tech-

TABLE III
Nucleic Acid Phosphorus Values for Rat Liver Reported in the Literature

| | Ribo-P | Desoxy-P | Ratio |
|---|---------|----------|-------|
| Schmidt & Thannhauser (13) | 87 | 26 | 3.3 |
| | 102 | 22.5 | 4.5 |
| Schneider (14) | 77.7 | 25.5 | 3.0 |
| Davidson (23) (200-240-g. rats) (60- 80-g. rats) | 77-110 | 21-25 | 4.0 |
| | 106-122 | 28-37 | 3.6 |

nique which allowed the entire analysis to be done in the same test tube, thereby obviating transfer losses. Their data show that with a total P of 328 they recovered only 307, which may be comparable to the losses shown in Table I, where many transfers were made. Euler (27) has recently proposed using exhaustive extractions in the Schmidt and Thannhauser method in an effort to avoid these discrepancies.

It apparently makes little difference whether the livers are perfused or the animals are bled by decapitation except in the acid-soluble fraction where values are slightly higher for the latter group. When no bleeding is done, as in Nos. 26 and 27, all the values are increased, especially the lipid phosphorus.

It can be seen from Table II that the individual values for ribo P vary more widely than those for desoxy P. This may be due to several

factors. Davidson and Waymouth (28), Kosterlitz (29, 30), and others have shown that the ribonucleic acid content of liver can be altered by fasting or varying the diet, while desoxyribonucleic acid changes but little. By using radioactive phosphorus, Brues, Tracy, and Cohn (31) and Hammarsten and Hevesy (32) have shown that the rate of turnover of ribonucleic acid is much greater than that of desoxyribonucleic acid. Shubnikova (33) has found a similar lability of the ribonucleic acid contrasted to a marked stability of desoxyribonucleic acid in protozoan cells.

It would appear then that several conclusions can be drawn from this work:

1. The methods available at the present time for the estimation of nucleic acids in tissues leave much to be desired.
2. The data presented in this paper follow the same pattern as similar data found in the literature.
3. From the data presented there is no significant change with age in any of the phosphorus-bearing fractions of liver studied.

It is perhaps not surprising that no fundamental age changes have been found in liver. Liver is a very "active" tissue; it continues to produce plasma protein at normal levels in old age, and retains great powers of regeneration. It may be, then, that liver remains a relatively young tissue even in the senile individual. Other tissues, such as cardiac muscle, might show significant changes. [Lowry *et al.* (11,12) determined total nucleic acid phosphorus in rat kidney, liver, brain, skeletal and cardiac muscle and found no significant change with age. Estimations of the individual nucleic acids were not done, however.] It must be kept in mind also that studies such as this give only indirect information. While no quantitative shift in the phosphorus content of the various fractions has been found, there may be important qualitative changes which are undetected.

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SUMMARY

The concentrations of acid-soluble, lipide, ribonucleic-acid, desoxyribonucleic-acid, and total phosphorus in the livers of young and old

mice have been investigated. The method of Schmidt and Thannhauser was used. No statistically significant change has been observed in any of these quantities.

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Studies on the Mechanism of Protein Synthesis in Embryonic and Tumor Tissues. I. Evidence Relating to the Incorporation of Labeled Amino Acids into Protein Structure in Homogenates¹

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INTRODUCTION

The relatively high activity of embryonic and tumor tissues in the *in vitro* uptake of C¹⁴-labeled amino acids (1,2) makes these systems attractive for studies of enzymic mechanisms of protein synthesis. The present exploratory study is concerned primarily with this uptake process in two rapidly growing tissues—fetal rat liver and mouse tumor.

The experiments with slices (1) and homogenates (3,4) of normal, adult mammalian liver appear to indicate that the labeled amino acids were incorporated into protein molecules. When carboxyl-labeled glycine was employed in the incubations, the radioactive protein subsequently isolated was found to yield no C¹⁴O₂ upon treatment with ninhydrin. Following hydrolysis of the protein by a combination of proteinases and peptidases, ninhydrin released most of the C¹⁴. After complete acid hydrolysis, all of the radioactivity of the protein was released as C¹⁴O₂ by ninhydrin. Additional evidence (4) for the incorporation of glycine, and also labeled leucine and lysine, into homogenate proteins was obtained by chromatographic analysis, which revealed radioactive peptides in partial acid hydrolysates.

Recently Keston² found that the major portion of the C¹⁴ incorporated by liver homogenates incubated with labeled glycine could be released by dialysis of the protein against ammonium hydroxide solutions. Greenberg and associates² confirmed this finding.

¹ Aided by a grant from the Iowa Division of the American Cancer Society.

² Personal communication.

The author of the present paper has observed that most of the C^{14} is likewise removed by dissolving the protein in dilute sodium hydroxide at room temperature, and then reprecipitating it with trichloroacetic acid (TCA). In contrast to this *in vitro* effect, liver protein prepared from rats injected with labeled glycine lost very little C^{14} when treated with alkali. This difference in behavior suggests that the C^{14} had been chiefly incorporated into a peptide fraction, and not protein molecules, in the *in vitro* experiments. The interpretation is somewhat complicated in that the alkali appears to cause a certain degree of mild hydrolysis of the protein.

It was of interest to test the effect of alkali on proteins derived from the incubation of homogenates of embryonic and tumor tissue with labeled glycine. Protein from homogenates of fetal rat liver was found to resemble that of adult liver *in vivo* in that alkali treatment removed no significant proportion of the C^{14} . Similarly in the case of homogenates of mouse tumor, most of the protein-bound C^{14} was resistant to sodium hydroxide, in contrast to the relatively alkali-labile C^{14} of protein from homogenates of normal mouse liver.

That glycine may be atypical in certain respects is indicated by experiments performed with labeled alanine. The latter amino acid was incorporated into protein in a comparatively alkali-resistant form in all systems tested, including homogenates of adult liver.

It was considered of value to test the action of ninhydrin on unhydrolyzed and completely hydrolyzed proteins from the relatively more active embryonic and tumor homogenates, as was done in previous studies with homogenates of normal adult liver, in order to determine whether these greater uptakes of labeled amino acids represented true incorporation of the latter into peptide combination.

EXPERIMENTAL

Labeled Amino Acids

Glycine-1- C^{14} and DL-alanine-1- C^{14} (carboxyl-labeled) with activities of 6.7 and 5.6 μ curies/mg., respectively, were purchased from Tracerlab Inc., on allocation from the U. S. Atomic Energy Commission. The glycine-1- C^{14} was diluted with 5 parts of inactive glycine, and the alanine-1- C^{14} with approximately 3 parts of DL-alanine, to give stock solutions (in distilled water) with specific activities of 98,000 and 138,000 counts/min./mg. (c/m/m), respectively. These activities were determined as follows:

A portion of each solution, containing exactly 0.5 mg. of amino acid, was mixed with 500 mg. of non-radioactive compound and the mixed material crystallized from a concentrated aqueous solution by the addition of alcohol. The crystals were dried

and powdered. Portions weighing 10–15 mg. were dispersed in about 3 ml. of acetone in a small Potter homogenizer. The suspension was poured quickly onto a tared aluminum disk (3.5 cm. diameter) seated in a "collecting unit."³ Then an additional 3 ml. of acetone was added with a rapid motion, to give a highly uniform dispersion of the amino acid. After evaporation of the acetone (at room temperature), the disk was carefully removed from its holder, placed in a 90°-oven for 1 hr., then reweighed, and the radioactivity of the amino acid layer (area = 7.02 cm.²) measured with a Geiger-Muller counter equipped with a 1½-in. diameter thin mica window. Samples were counted to within a probable error of 2%, and were corrected for background, and for self-absorption of radiation by reference to a table of corrections for varying layer thicknesses. Replicate samples generally agreed to within 3%. The average value for the c/m/m was multiplied by 1000 to give the c/m/m for the stock radioactive solution.

Preparation of Homogenates

Fetal livers were obtained from Sprague-Dawley rats pregnant for 18–19 days. The female was killed by a blow on the head. The embryos were quickly removed and their livers dissected out. Approximately 1.5–2 g. of fetal livers was obtained from one mother. This material was ground for 1 min. with 1 vol. of ice-cold 0.9% KCl–0.4% KHCO₃ solution of pH 7.5 (saturated with 95% O₂–5% CO₂) in a glass homogenizer. The latter was immersed in ice water during the grinding.

The same homogenizing technique was used with livers of young, adult male rats or mice, and with mouse tumors. The latter were fourth generation transplants from a spontaneous mammary carcinoma. They were of moderate size (2–4 g.), with almost no necrotic material.

Incubation Procedure

A series of 12-ml. thick-walled centrifuge tubes were prepared, each containing either 0.19 mg. of glycine-1-C¹⁴ or 0.45 mg. DL-alanine-1-C¹⁴ (dried from aqueous solution).

To each tube was added 0.25 ml. of freshly-prepared homogenate. The tube was flushed thoroughly with 95% O₂–5% CO₂ gas, quickly stoppered, and inserted in a horizontal position into a cylindrical drum submerged in a 37° bath. The drum was rotated at 10 r.p.m., so that the homogenates flowed continuously around the inner walls of the tubes.⁴

The glycine was employed at 0.01 *M*, and the DL-alanine at 0.02 *M* concentration in the homogenates.

Separation of Radioactive Protein

After a definite incubation period, each tube was removed from the bath and its contents diluted with 1 ml. of water, followed by 5 ml. of 10% TCA. The precipitated

³ This apparatus is prepared from a wide-mouth jar with a flat metal screw cap (3.7 cm. inside diameter). The rim of the jar is ground flat and polished with emery powder. Then the upper portion is cut off to give a ring about 3 cm. high. A 1.5-cm. circular hole is cut in the center of the cap. The aluminum disk is inserted in the cap, and the latter screwed tightly to the glass ring. The unit is inverted on a flat surface.

⁴ The drum was inclined slightly, so that the homogenates remained in the lower portions of each tube.

protein was centrifuged, and washed three times with 6 ml. of 5% TCA, centrifuging each time. Prior to the last centrifugation, the tube was heated for 15 min. at 90° to remove nucleic acids (5). Then the protein was extracted once with 6 ml. of alcohol, twice with hot 3:1 alcohol-ether and once with ether. After decanting the ether, the tube was laid horizontally, and tapped to spread out the protein, during evaporation of the ether. A light-colored, powdery material was obtained by this technique. It contained 15.1% nitrogen.

Measurement of Radioactivity of Proteins

The technique was similar to that described for amino acids, except that the protein of each tube was dispersed in ether (instead of acetone), and after pouring the suspension into a collecting unit (containing an aluminum disk), four volumes of petroleum ether were added.⁵ The radioactivities of the dried layers (weighing 12–13 mg.) were generally in the order of 40–200 counts/min. (4–20 times background), so that a counting error of less than 3% was readily achieved.

Methods of Expressing C¹⁴ Incorporation

The activities of different systems in taking up a labeled amino acid were generally compared in terms of the radioactivities of the isolated proteins, expressed as c/m/m.

However, in comparing two different amino acids, the C¹⁴ uptakes were expressed in micrograms of labeled carbon per gram protein ($\mu\text{g.}/\text{g.}$), and were thus independent of the specific radioactivities of the glycine and alanine, as well as the identity of the labeled amino acid residues in the proteins. From the c/m/m values of the glycine-1-C¹⁴ (98,000) and alanine-1-C¹⁴ (138,000) used in the incubations, and their percentages of labeled carbon,⁶ 1 $\mu\text{g.}$ of the latter corresponds to 613 counts/min. in the case of glycine, and 1020 counts/min. for alanine. The counts/min./g. protein divided by these values gave the micrograms of labeled carbon per gram protein.

Ninhydrin Treatment of Proteins and Protein Hydrolysates

The U-tube apparatus of Van Slyke, McFadyen, and Hamilton (6) was used, except that 1 ml. of 1 *N* NaOH (CO₂-free) in a 12-ml. centrifuge tube was substituted for the flask containing Ba(OH)₂. The flask at the other arm contained 8 mg. of protein, 2 ml. of water, 100 mg. ninhydrin, 50 mg. pH 4.7 citrate buffer, and 6 mg. inactive glycine (to provide CO₂). When hydrolysates were used, the glycine was omitted. Hydrolysis was performed by heating 40 mg. protein with 0.7 ml. of 6 *N* HCl in a sealed tube for 18 hr. at 116°. The hydrolysate was neutralized and made up to 10 ml. Then 2-ml. aliquots (equivalent to 8 mg. protein) were taken for the analysis with ninhydrin.

After a 12-min. heating period (at 100°), the CO₂ trapped in the alkali was precipitated as BaCO₃ by adding 1 ml. of 1 *M* BaCl₂. The precipitate was centrifuged,

⁵ A highly quantitative transfer of the protein was unnecessary, since only its *specific* radioactivity was measured. If the petroleum ether was omitted, the protein layer cracked upon drying.

⁶ The ratios of C¹⁴ to C¹² for the carboxyl carbons were sufficiently low to permit the use of 12 as an adequately accurate weight for this carbon atom.

washed with 3 ml. of 50% alcohol, then with absolute alcohol, and finally resuspended in acetone and transferred to a weighed aluminum plate in a collecting unit. After drying in air, the plate was reweighed, and the radioactivity of the BaCO_3 layer measured. Corrections for self-absorption were made, using the data of Yankwich, Norris, and Huston (7). Replicate determinations on protein hydrolysates usually agreed to within 2-3%.

Treatment of Proteins with Alkali

Fifty mg. of protein was dissolved (by prolonged stirring) in 2 ml. of 0.5 *N* NaOH at 26-28° in a tared 12-ml. centrifuge tube. After 2 hr. 5 ml. of 10% TCA was added with stirring. The precipitate was centrifuged, washed once with 3 ml. of 5% TCA, and lastly with 3 ml. of water at 0°. After the last centrifugation, the precipitate was lyophilized, and the tube reweighed. Quadruplicate portions of the dry protein were collected on aluminum plates for counting.

RESULTS AND DISCUSSION

Rates of C^{14} Incorporation into Homogenates Proteins

Figure 1 illustrates the relatively high activity of fetal, as compared to adult rat liver, in the uptake of glycine-1- C^{14} and alanine-1- C^{14} . If comparisons are made for a 2.5-hr. period, it is seen that fetal liver is

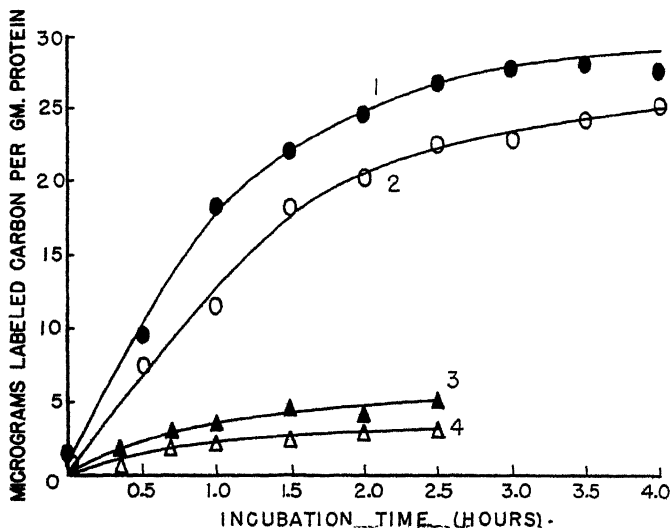


Fig. 1. Rates of C^{14} -incorporation into protein of rat liver homogenates. Curves 1 and 3: Fetal and adult liver, respectively, with glycine-1- C^{14} . Curves 2 and 4: Fetal and adult liver, respectively, with alanine-1- C^{14} .

approximately six to seven times as active as adult liver homogenate, with either glycine-1- C^{14} or alanine-1- C^{14} . A similar differential was observed by Zamecnik and co-workers, who found that fetal rat liver slices were six times as active as adult slices in the incorporation of alanine-1- C^{14} , in 3.5-hr. incubation experiments (1).

A comparison of mouse tumor with normal mouse liver (Fig. 2) shows that, in 3 hr., the protein of the tumor homogenate incorporated five to six times as much glycine-1- C^{14} or alanine-1- C^{14} into liver protein. A similar observation (1), that hepatoma slices are seven times as active as normal rat liver slices in the incorporation of alanine-1- C^{14} , has been reported.

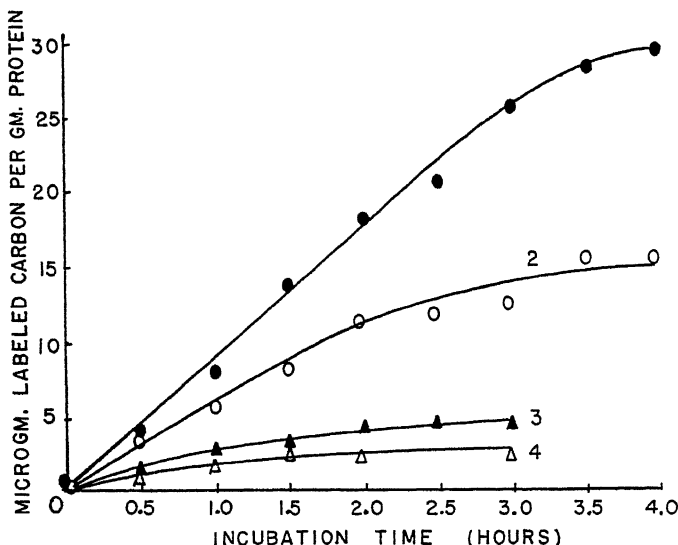


Fig. 2. Rates of C^{14} -incorporation into protein of mouse tissue homogenates. Curves 1 and 3: Tumor and normal adult liver, respectively, with glycine-1- C^{14} . Curves 2 and 4: Tumor and adult liver, respectively, with alanine-1- C^{14} .

With each type of homogenate, 0.01 M glycine-1- C^{14} consistently gave rise to higher C^{14} concentrations in protein than did 0.02 M DL-alanine-1- C^{14} . The same conclusion was reached (1) in tissue-slice experiments with normal liver and hepatoma. This difference may be related to the fact that part of the glycine-1- C^{14} is converted to serine-1- C^{14} *in vitro* (8), and the latter compound augments the radioactivity

of liver protein. It is not yet known whether alanine-1-C¹⁴ forms significant quantities of other amino acids in homogenates.

From the labeled carbon incorporated, it is possible to estimate the degrees of turnover of glycine and alanine in the proteins. The 17.5 μ g. of labeled carbon taken up by 1 g. of fetal liver protein in 1 hr. (Fig. 1) corresponds to 0.11 mg. glycine (including labeled serine in this calculation). Assuming 5% glycine in the protein, the initial rate of turnover of glycine residues was $0.11/50 \times 100$ or 0.22%/hr. The corresponding estimate for alanine is 0.19%. These values are approximately half as great as those found with fetal liver slices (1). The replacement values calculated for adult rat liver homogenate are much lower: 0.04% for glycine and 0.03% for alanine.

The initial turnover rates for mouse tumor homogenates (Fig. 2) were 0.12 and 0.09 for glycine and alanine, respectively, while the corresponding values for normal adult mouse liver were 0.04 and 0.03, the same as with rat liver homogenate. Borsook and co-workers (4)

TABLE I

Treatment of Radioactive Proteins and Protein Hydrolysates with Ninhydrin

The homogenate proteins represent pooled material from 15 to 25 individual experiments, including those referred to in Fig. 1. The values recorded are averages of triplicate or quadruplicate determinations.

| Source of protein | Labeled amino acid employed | C ¹⁴ incorporated into protein | C ¹⁴ released from protein by ninhydrin | C ¹⁴ released from hydrolyzed protein by ninhydrin |
|--|-----------------------------|---|--|---|
| | | <i>c/m/m^b</i> | <i>c/m/m^b</i> | <i>c/m/m^b</i> |
| Rat liver, <i>in vivo</i> ^a | Glycine-1-C ¹⁴ | 16.1 | 0.1 | 15.8 |
| | Alanine-1-C ¹⁴ | 5.9 | 0.0 | 6.05 |
| Fetal rat liver homogenate | Glycine-1-C ¹⁴ | 18.55 | 0.2 | 18.15 |
| | Alanine-1-C ¹⁴ | 17.3 | 0.1 | 16.65 |
| Rat liver homogenate | Glycine-1-C ¹⁴ | 3.25 | 0.1 | 3.15 |
| | Alanine-1-C ¹⁴ | 2.50 | 0.0 | 2.35 |
| Mouse liver homogenate | Glycine-1-C ¹⁴ | 3.65 | 0.0 | 3.65 |
| | Alanine-1-C ¹⁴ | 2.40 | 0.05 | 2.30 |
| Mouse tumor homogenate | Glycine-1-C ¹⁴ | 19.4 | 0.25 | 18.4 |
| | Alanine-1-C ¹⁴ | 10.9 | 0.0 | 10.95 |

^a Approximately 2 mg. of glycine (containing 200,000 counts/min. of C¹⁴) or alanine (280,000 counts/min.) were injected intraperitoneally into a 100 g. male. The animal was sacrificed after 18 hr.

^b Counts/minute/milligram.

reported an unusually high turnover of lysine in protein, 0.15%/hr., for *adult* guinea-pig liver homogenate incubated at 38° with L-lysine-6-C¹⁴ (about 0.01 *M* concentration). They state that glycine-1-C¹⁴ and L-leucine-1-C¹⁴ are incorporated at much lower rates by this homogenate system.

Results of Ninhydrin Treatments

Table I shows that none of the proteins from either *in vivo* or *in vitro* sources released significant amounts of C¹⁴O₂ when treated with ninhydrin. Following acid hydrolysis, essentially all of the radioactivity in each case was accounted for as C¹⁴O₂, following ninhydrin treatment.

TABLE II

Treatment of Radioactive Proteins with Alkali

A 50-mg. portion of each protein referred to in Table I was treated with NaOH as described in the experimental section. The values in columns 3 and 4 represent the average counts for triplicate plates.

| Source of protein | Labeled amino acid employed | Radioactivity of protein | Radioactivity of protein after NaOH treatment | Weight of recovered protein |
|----------------------------|-----------------------------|--------------------------|---|-----------------------------|
| | | <i>c/m/m^a</i> | <i>c/m/m^a</i> | <i>mg.</i> |
| Rat liver, <i>in vivo</i> | Glycine-1-C ¹⁴ | 16.1 | 15.45 | 49.1 |
| | Alanine-1-C ¹⁴ | 5.9 | 5.45 | 49.2 |
| Fetal rat liver homogenate | Glycine-1-C ¹⁴ | 18.55 | 17.45 | 50.5 |
| | Alanine-1-C ¹⁴ | 17.3 | 17.5 | 49.2 |
| Rat liver homogenate | Glycine-1-C ¹⁴ | 3.25 | 0.60 | 49.0 |
| | Alanine-1-C ¹⁴ | 2.50 | 2.20 | 50.8 |
| Mouse liver homogenate | Glycine-1-C ¹⁴ | 3.65 | 0.75 | 50.3 |
| | Alanine-1-C ¹⁴ | 2.40 | 2.20 | 48.4 |
| Mouse tumor homogenate | Glycine-1-C ¹⁴ | 19.4 | 14.65 | 49.9 |
| | Alanine-1-C ¹⁴ | 10.9 | 9.25 | 48.8 |

^a Counts/minute/milligram.

It can be concluded with considerable confidence that virtually 100% of the C¹⁴ in each protein represents carboxyl-labeled amino acid residues bound in peptide linkages.

Effect of Alkali on Proteins

Examination of the last column in Table II indicates that the recovery of protein was almost quantitative in all cases. While the NaOH

treatment did not affect the proteins with respect to their precipitability by TCA, it was observed that the TCA precipitates were soluble in absolute ethanol, unlike the original proteins. Because of this change in properties, it was necessary to use the washing and lyophilizing technique described in the experimental section, in order to reisolate and dry the protein.

Comparison of columns 3 and 4 in Table II shows that the proteins from *in vivo* experiments with labeled glycine and alanine had 96 and 92%, respectively, of their initial C^{14} concentrations after alkali treatment. Similarly, the protein derived from fetal liver homogenate incubated with either glycine-1- C^{14} or alanine-1- C^{14} retained virtually all of its C^{14} upon alkali treatment.

When adult rat or mouse liver homogenate was incubated with alanine-1- C^{14} , the protein retained approximately 90% of its original specific activity. This compares favorably with the *in vivo* protein. However, the proteins from the same homogenates, but with glycine-1- C^{14} , retained only one-fifth of their initial C^{14} concentrations. Inasmuch as the recovery of protein was almost quantitative, the C^{14} removed by alkali in these experiments was concentrated in a small fraction (presumably a peptide) of relatively high specific radioactivity.

SUMMARY

When homogenates of fetal rat liver or mouse tumor were incubated with glycine or alanine labeled with C^{14} in the carboxyl group, the uptake of C^{14} by protein was several times as great as when homogenates of adult rat or mouse liver were employed. The turnover of glycine or alanine residues in the embryonic and tumor homogenates was of the order of 0.1–0.2%/hr.

No C^{14} was released from any of the homogenate proteins by heating with ninhydrin. Following complete acid hydrolysis of the proteins, virtually all of the C^{14} was recovered as $C^{14}O_2$ upon ninhydrin treatment. This indicates that the C^{14} in the proteins was due to amino acids combined in peptide linkage.

It was found that the protein from adult liver homogenate, incubated with labeled glycine, lost most of its C^{14} upon being dissolved in dilute alkali. The available evidence indicates that the C^{14} was concentrated in a labile peptide fraction, associated with the protein. Fetal liver and tumor homogenate proteins retained their C^{14} when treated with alkali.

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Distribution of Tracer Carbon Among the Lipides of the Alga *Scenedesmus* During Brief Photosynthetic Exposures

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INTRODUCTION

Brown, Fager, and Gaffron (1) reported that the benzene-soluble fraction of *Scenedesmus obliquus* becomes radioactive during half-minute photosynthetic exposures to $C^{14}O_2$. With longer tagging periods, the proportion of assimilated tracer in this fraction as well as in the insoluble "C" residue increased at the expense of the water-soluble fraction. The tracer fixed anaerobically in darkness remained almost completely water-soluble. When the algae were exposed to $C^{14}O_2$ aerobically in darkness, the tracer rapidly entered all three solvent fractions, but the amount incorporated was less than 2% of that fixed during an equivalent period in saturating light.

Distinctive properties were reported for the water-soluble compounds which were tagged during brief exposures in light and in darkness. The tracer carbon which was assimilated in darkness was rapidly removed from the water-soluble fraction by subsequent dark metabolism; the tracer which was incorporated in the water-soluble fraction by photosynthesis, by contrast, remained in this fraction when the algae were kept in darkness, and was removed only on exposure to light. These observations led to the conclusion that the tracer incorporated in water-soluble substance by photosynthesis was in a stable photosynthetic intermediate, unavailable to the dark metabolism of the cell, and capable only of photochemical transformation.

The latter conclusion, in addition to those summarized by Kamen

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(4) and Fager (3), is in almost complete disagreement with those of Benson *et al.* (5). The present study of the location of tracer carbon in the benzene-soluble constituents after 40 sec. exposure was undertaken with the ultimate objective of resolving this issue.

MATERIALS AND METHODS

Scenedesmus obliquus was grown and tagged under previously described conditions (2,3). After exactly 40 sec. exposure to dilute $\text{Na}_2\text{C}^{14}\text{O}_3$ in light, the tagged algal suspension was poured into a large volume of boiling water. The water-insoluble solids were recovered on the centrifuge, and were extracted six additional times with distilled water. The fats and pigments were extracted from the residue with 85% methanol, 80% acetone, methanol, acetone, and benzene—essentially the same method as was employed by Milner on *Chlorella* (6). The combined extract was washed with water, and the organic solvent phase was evaporated to dryness at low temperature. The yield was 21% of the total algal solids, and the specific radioactivity of different preparations ranged from 60–200 counts/min./mg.

The pigments were separated on MgO (7,8) and confectioners' sugar columns (9,10). Saponification of the fats was effected with boiling methanol containing 20% KOH, as well as with 5% sodium ethylate (11,12). The saponification products were separated into unsaponifiable, fatty acid, and water-soluble fractions prior to steam distillation. The nonvolatile fatty acids were separated into saturated and unsaturated fractions by a micro adaptation of the lead salt-alcohol method (11,13). The water-soluble saponification products were oxidized to carbon dioxide with chromic acid. The radioactivity measurements on organic solids and BaCO_3 were made with an internal sample flowing-gas-counter. Appropriate corrections were applied for background and self-absorption.

RESULTS

Proof That "A" Fraction Radioactivity Does Not Result From Contamination

The specific activity of the water-soluble or "B" fraction was approximately 200 times higher than that of the benzene-soluble or "A" fraction. If allowance is made for differences in specific activity within the water-soluble fraction, it is apparent that contamination with mere traces of "B" by co-solution could account for all of the "A" fraction radioactivity. The following experiments demonstrate that the radioactivity of the "A" fraction does not result from such contamination.

1. Three aliquots of a petroleum-ether solution, each containing 6.9 mg. "A" solids and 1440 counts/min., were evaporated at low temperature and dissolved in 20 ml. benzene, 20 ml. ethyl ether, and 20 ml. *n*-hexane, respectively. Twenty milliliters of water was added to each of these solutions, and the stoppered vessels were shaken

TABLE I
*Distribution of "A" Fraction Solids and Radioactivity
 Between Organic Solvents and Water*

| Organic solvent | Recoveries from organic solvent phase | | Recoveries from water phase |
|------------------|---------------------------------------|--------------------|-----------------------------|
| | Solids | Radioactivity | |
| | <i>mg.</i> | <i>counts/min.</i> | |
| Benzene | 6.9 | 1475 | zero |
| Ethyl ether | 7.2 | 1490 | zero |
| <i>n</i> -Hexane | 6.9 | 1400 | zero |

intermittently for 3 hr. The phases were allowed to separate, and the solids and radioactivity in each phase were determined on 15-ml. aliquots. No solids or counts were recovered from the aqueous phase. The solids and counts recovered from the organic solvents showed less than 5% variation from that of the original extract (Table I).

2. Following the procedure of MacKinney (14), an ether solution of "A" solids was washed with water twelve times by allowing it to rise from a fine capillary jet through a 10-cm. layer of distilled water, the water being changed between each extraction. The specific activity of the "A" solids was the same before and after twelve of these treatments.

Composition of "A" Fraction

Alpha- and β -carotene fractions were isolated by developing MgO columns with petroleum ether in an atmosphere of nitrogen. The absorption maxima, measured in pure *n*-hexane with a Beckman spectrophotometer, were 4430 Å and 4710 Å for the α -, and 4475 Å and 4750 Å for the β - fractions. The α - and β -carotene contents were calculated from the absorption observed at 4430 Å and 4475 Å, employing specific absorption coefficients of 272 and 258 respectively (15). The total carotene content was 0.29% of the "A" solids or 0.06% of the total algal solids. The ratio of the α : β fractions was 1:3.

The more strongly adsorbed carotenol did not separate into components during lengthy development of MgO columns with petroleum ether containing up to 5% acetone. The carotenol showed absorption maxima at 4420–4450 Å and at 4700–4720 Å in absolute ethanol. Assuming an absorption coefficient of 255 at 4450 Å, the carotenol content was 0.39% of the "A" solids or 0.08% of the total algal solids.

The chlorophyll content, determined by the method of Comar and Zscheile (16,17), was 9.96% of the total "A" solids or 2.09% of the total dry weight. The total content of chlorophylls and carotenoids thus

amounted to 10.63% of the "A" fraction or 2.24% of the total algal solids.

The distribution of the remaining "A" solids after saponification was approximately 14% unsaponifiable, 51% fatty acid, and 20% water-soluble. The fatty acids were predominately unsaturated. Milner (6) has reported that the saturated and unsaturated fatty acids of *Chlorella* are almost exclusively C_{16} and C_{18} . Bergmann and Feeney (18) found that the unsaponifiables of *Scenedesmus obliquus* are mainly hydrocarbons and higher aliphatic alcohols. Sterols (chiefly chondrillasterol) account for 10–20% of the unsaponifiable fraction (18).

*Apparent Association of Radioactivity With
the Chloroplast Pigments*

The "A" solids were fractionated with MgO–Celite under conditions similar to those employed by Zscheile and Whitmore (7). Approximately 40 mg. "A" solids in 20 ml. petroleum ether was drawn into the MgO column (15×1.8 cm.) and extraction with petroleum ether was continued, with head pressure and suction, until 100 ml. colorless eluate was collected. The column was next extracted with 25 ml. 10% acetone in petroleum ether, which eluted the carotene. The carotenol was eluted with 100 ml. 60% acetone in petroleum ether. The column was then extruded. The chlorophylls and fatty materials were recovered by extracting the magnesium oxide eight times with 50-ml. volumes of hot 80% acetone in water. The extracted adsorbent was colorless and did not exhibit radioactivity. Fractionation on the compound $\text{Ca}(\text{OH})_2$ –MgO column employed by Bernstein and Thompson (8) resulted in the same distribution of radioactivity as was obtained by the foregoing method, the only difference being the retention of the chlorophyll and fatty substances in the narrow $\text{Ca}(\text{OH})_2$ layer.

The distribution of radioactivity and solids among the four fractions obtained with magnesium oxide is shown in Table II. Approximately 5% of the radioactivity appeared in each of the three fractions eluted with petroleum ether and petroleum ether–acetone. The greater part of the radioactivity and solids was recovered in the fourth fraction which contained all of the chlorophyll as well as most of the non-pigments. The specific activity of this fraction was slightly lower than that of the original "A" solids. The weight of the eluted carotenoid fractions exceeded the carotenoid content as measured with the spec-

trophotometer. This procedure thus provided carotene and carotenol fractions free of chlorophyll, but the chlorophyll was not separated from the fatty substances comprising the bulk of the "A" fraction. Some contamination of the carotenoids with non-pigments was also indicated by the solids data. The specific activity values for the four fractions obtained with magnesium oxide were quite similar to the original value for the unfractionated "A" solids.

TABLE II
Fractionation of Radioactivity and Solids on Magnesium Oxide

| | Eluting solvent | Total activity | | Specific activity | Solids |
|-------------------------------|----------------------------------|---------------------|----------|------------------------|-------------|
| | | counts/min. 6280 | % 100 | counts/min./mg. 129 | mg. 42.4 |
| Total solids | | | | | |
| "Lipide" | Petroleum ether | 317 | 5.1 | 264 | 1.2 |
| Carotene | Petroleum ether + 10% acetone | 470 | 7.5 | 214 | 1.5 |
| Carotenol | Petroleum ether + 60% acetone | 390 | 6.2 | 97.5 | 3.9 |
| Chlorophyll + non-pigments | 80% acetone, 20% water | 4430 | 70.6 | 109 | 40.5 |

The carotene, carotenol, and "crude chlorophyll" fractions were readsorbed on freshly prepared individual MgO columns, and again recovered with the solvents indicated in Table II. The accompanying losses of radioactivity and solids ranged from 0-10%. So long as magnesium oxide was employed as the adsorbent, the chlorophyll remained associated with the greater part of the solids and radioactivity.

The carotene was also subjected to repeated adsorption and elution, employing the technique of Zscheile and his collaborators (15). After thorough development of a 6-in. magnesium oxide column with petroleum ether in a nitrogen atmosphere, the carotene was eluted with a minimum of 10% acetone in petroleum ether. After measuring the radioactivity, the carotene was readsorbed from petroleum ether on a further 6-in. column of magnesium oxide. The column was extracted with petroleum ether and 10% acetone in petroleum ether until the 0.5-in. carotene band had descended through half the column. The column was extruded and the carotene zone was cut out and eluted with acetone in a centrifuge tube. After measuring the radioactivity, the process was repeated on a third column. The counts/minute for the successively recovered carotene samples were 464, 164, and 104. Although a nitrogen atmosphere was employed as much as possible in these manipulations, bleaching of the carotene prevented further work with it. The solids content was not reduced

(1.7, 2.4, 1.8 mg.). The decrease in radioactivity which was observed in the successive samples suggests that more than half of the radioactivity in the carotene fraction is caused by impurities. Larger scale experiments with additional precautions would be required, however, to settle this point with certainty. The carotene was not completely freed of radioactivity under the foregoing conditions; this was also true of carotenoids separated from the "unsaponifiables," by crystallization and chromatography. From these observations, it is concluded that the carotenoids which comprise less than 1% of the present "A" fraction do not contain more than 5% of its total radioactivity.

Willstätter and Stoll's well known procedure (19), as well as carbohydrate adsorption columns, was investigated as a means of separating the chlorophyll from the fatty constituents. On subjecting 0.4 g. of untagged "A" solids to the Willstätter-Stoll (W-S) procedure, it was found that the chlorophyll lost its fluorescence but was not precipitated from light petroleum ether. Since it is this property on which the purification of chlorophyll very largely depends, the W-S method appeared unsuitable for the present material.

Exploratory work with untagged material showed that the chlorophyll in 10–20-mg. "A" samples could be separated into *a* and *b* zones on 10-in. columns of starch, sugar, or sorbitol.² Petroleum ether containing different concentrations of acetone and benzene was employed in developing the columns. Pure benzene, followed by 50% benzene in petroleum ether, was used to remove carotenol from the chlorophyll (10). Potato starch had the lowest, and sorbitol the highest adsorption capacity, as shown by band width and ease of elution. The "A" fraction solids adsorbed in the broad chlorophyll bands was reduced from ca. 90%, as obtained with magnesium oxide, to less than 50% on powdered sucrose. Contamination of the chlorophyll with colorless constituents thus had been reduced, but by no means overcome.

Ten milliliters of a petroleum-ether solution containing 13.4 mg. "A" solids, 828 counts/min., was drawn into a 10-in. powdered-sucrose column. The column was developed with 75 ml. petroleum ether, 50 ml. 2% acetone in petroleum ether, and 200 ml. 33% benzene in petroleum ether. The column was extruded, and the chlorophyll *a* and *b* zones were cut out and completely eluted with acetone (Table III, first sugar column). Although a much smaller amount of "A" solids was eluted with the chlorophyll from sucrose than from magnesium oxide (Table II), the weight of eluted solids (5.7 mg.) was still far greater than the chlorophyll content (1.3 mg.). The crude chlorophyll fraction eluted from magnesium-oxide columns showed approximately 70% of the total radioactivity (Table II). The crude chlorophylls eluted from sugar showed less than 25% of the total radioactivity (Table III).

The chlorophylls from the first sugar column were then adsorbed separately on two freshly prepared sugar columns. The columns were

² The hygroscopicity of sorbitol renders it unsuitable for general use as an adsorbent.

each developed with 50 ml. petroleum ether and 25 ml. 33% benzene in petroleum ether. The chlorophylls *a* and *b* both became concentrated in deeply colored 1-cm. bands, above which there was a diffuse, weakly colored zone having a depth of 1.5 in. The columns were extruded and the colorless and weakly colored zones were carefully separated from

TABLE III
Fractionation of Radioactivity and Solids on Powdered Sucrose

| | Eluting solvent | Total activity | Specific activity | Solids |
|----------------------------|---|----------------|-------------------|--------|
| | | counts/min. | counts/min. / mg. | mg. |
| First sugar column | | | | |
| Total solids | — | 828 | 62 | 13.4 |
| Carotene and non-pigments | Petroleum ether, 75 ml. | 75 | 50 | 1.5 |
| Carotenol and non-pigments | Two per cent acetone in petroleum ether, 50 ml. 33% benzene in petroleum ether, 200 ml. | 478 | 177 | 2.7 |
| Crude chlorophyll <i>a</i> | Acetone | 114 | 32 | 3.6 |
| Crude chlorophyll <i>b</i> | Acetone | 64 | 31 | 2.1 |
| Second sugar column | | | | |
| Chlorophyll <i>a</i> | Acetone | 0 | 0 | 1.0 |
| Diffuse zone | Acetone | 129 | 43 | 3.0 |
| Third sugar column | | | | |
| Chlorophyll <i>b</i> | Acetone | 0 | 0 | 0.8 |
| Diffuse zone | Acetone | 33 | 37 | 0.9 |

the well-defined chlorophyll *a* and *b* bands. The purified chlorophylls were completely free of radioactivity (Table III, second and third sugar columns). The radioactivity, as well as the remaining "A" solids which were adsorbed on these columns, was recovered in the weakly colored zones above the chlorophyll bands. On repeating this experiment in its

entirety, chlorophylls *a* and *b* were again obtained completely free of radioactivity.

As an upper limit, not more than 1% of the "A" fraction activity could have been in the purified chlorophylls without being detected in this study. Chlorophylls, in common with algal constituents in general, are synthesized during the usual tagging experiments. The synthesis of chlorophyll is separated from carbon dioxide assimilation by such a lengthy series of reactions that negligible amounts of C¹⁴ become incorporated in the chlorophyll during 40-sec. exposures.

Distribution of Radioactivity and Solids After Saponification

One hundred and thirty-seven milligrams of "A" solids, specific activity 205, was dissolved in 10 ml. methanol containing 20% KOH. The solution was refluxed for 2 hr. in an atmosphere of nitrogen. Ten milliliters water was added to the cooled solution, which was then evaporated to 10 ml. in nitrogen. The strongly alkaline solution was extracted exhaustively with ethyl ether. The "unsaponifiables" thus removed were subjected to a second 2-hr. saponification treatment, in which boiling 20% KOH in methanol was again employed. Twenty-five milligrams of solids, having a specific activity of 137, was recovered as "unsaponifiables" (Table IV). These solids were subsequently dissolved

TABLE IV
Distribution of "A" Fraction Radioactivity Accompanying Saponification

| | Solids | Total | Specific activity | Per cent of total "A" fraction radioactivity |
|----------------------------------|--------|-------------|-------------------|--|
| | mg. | counts/min. | counts/min./mg. | |
| "A" solids before saponification | 137 | 28,100 | 205 | 100 |
| Unsaponifiable | 25.0 | 3,425 | 137 | 12 |
| Fatty acid, nonvolatile | 75.2 | 17,105 | 234 | 61 |
| Fatty acid, volatile | 4.3 | 245 | 57 | 0.9 |
| Water-solubles, nonvolatile | 22.5 | 5,810 | 258 | 21 |
| Water-solubles, volatile | 3.9 | 244 | 62.5 | 0.9 |
| Insoluble residue | 6.1 | 166 | 27 | 0.6 |

in 10 ml. of 5% sodium ethylate which was brought to boiling under reflux in nitrogen and then allowed to cool gradually to room temperature. Twenty and two-tenths milligrams of solids having a specific activity of 156 were recovered as "unsaponifiable" after this treatment. On adjusting the aqueous "sodium ethylate" solution to pH 2.0 and again extracting with ethyl ether, 4.5 mg. additional solids having a specific activity of 58 were recovered. The retention of over 3000 counts in the unsaponified fraction after these three drastic saponification treatments is considered to establish that compounds in this category contain C^{14} after 40-sec. exposures.

Carotenoids and phytol account for 20% of the unsaponifiable solids. Experiments on chlorophyll reported in the preceding section indicate that the contribution of phytol to the radioactivity of this fraction is negligible. The contribution of the carotenoids should be appreciable on the grounds that these compounds were not obtained free of radioactivity by chromatography. It was observed that most of the carotenoids were precipitated out of a 60% ethanol-water solution of the unsaponifiables which was stored a few hours at 0° C. The precipitate was centrifuged out and washed once with cold 60% ethanol. It was then redissolved in warm absolute ethanol which was next diluted with 35% water. Yellow crystals again separated on storing at 0° C.; yield 1.5 mg., total counts/min. 68. This experiment indicates that the unsaponifiable radioactivity is mainly in colorless constituents.

When the concentrated potassium hydroxide solution had been freed of unsaponifiables and adjusted to pH 1.0 with HCl, a voluminous precipitate rose to the surface. Exhaustive extraction with ethyl ether removed 79.5 mg. solids and 17,350 counts/min. radioactivity (Table IV). This fraction thus contained 58% of the total "A" solids and 62% of the total "A" radioactivity. A small amount of solids could not be dissolved in either water or ethyl ether. This material was almost free of C^{14} (6.1 mg., specific activity 27).

The water-soluble saponification products were subjected to steam distillation at pH 1.5, and the nonvolatiles were then oxidized to CO_2 with hot chromic acid. The CO_2 was continuously swept out of the refluxing apparatus with nitrogen, bubbled through 0.5% sulfuric acid, and then absorbed in dilute sodium hydroxide. The barium carbonate precipitate obtained on adding $BaCl_2$ and NH_4Cl showed a total activity of 5810 (Table V). Before saponification, the "A" fraction was nonvolatile, soluble in ethyl ether, and did not contain either water-soluble solids or water-soluble radioactivity (Table I); after saponification, approximately 20% of the "A" solids and radioactivity was soluble in water and insoluble in ethyl ether (Table IV).

The "A" fraction was separately tested for free fatty acid before saponification by thoroughly extracting a petroleum ether solution (70 mg. solids, 14,350 counts/min.) with 0.02 *M* NaOH. The aqueous extract was then acidified to pH 1.5 and extracted with ethyl ether. No radioactivity and less than 1 mg. of solids was recovered. The crude fatty acid fraction recovered after saponification, which accounted for 58% of the solids and 62% of the C^{14} , was therefore a product of saponification. The radioactivity in this major fraction was reduced less than 2% by 2 hr. rapid steam distillation at pH 1.5 (Table IV).

TABLE V
Distribution of Fatty Acid Radioactivity Between the Saturated and Unsaturated Components

| | Solids | Specific activity | Total activity |
|------------------------|--------|-------------------|----------------|
| | mg. | counts/min./mg. | counts/min. |
| Crude fatty acid | 69 | 220 | 15,180 |
| Saturated fatty acid | 23 | 256 | 5,890 |
| Unsaturated fatty acid | 39.5 | 133 | 5,250 |

Sixty-nine milligrams nonvolatile fatty acids were subjected to the following micro adaptation of Twitchell's lead salt-alcohol method (11,13). The solids were transferred to a small centrifuge tube with ether, evaporated to dryness, and dissolved in 1 ml. 95% ethanol. One hundred milligrams lead acetate $[Pb(C_2H_3O_2)_2 \cdot 3H_2O]$ was separately dissolved in 1 ml. 95% ethanol. Both solutions were brought just to boiling, mixed, brought to boiling again, and cooled rapidly to 10°C. After 3 hr. at 10°C., the suspension was centrifuged, and the supernatant, which gave a positive test for residual lead, was removed to a second vessel. The precipitate was washed twice in 2 ml. cold 95% ethanol by resuspending and centrifuging. The 4 ml. of wash liquid was combined with the supernatant fraction. The precipitate was dissolved in 2 ml. hot 95% ethanol containing 0.5% acetic acid, cooled to 15°C., and stored 2 hr. at that temperature. The reprecipitated lead soap was centrifuged and washed twice with cold 95% ethanol. The mother liquor and wash liquids from the second precipitate were combined with those from the first. The lead precipitate was next dispersed in 1.5 ml. ethyl ether and decomposed with 0.5 ml. concentrated nitric acid. Ten milliliters ether was added, and the ether phase was washed with distilled water until the washings were neutral to litmus. The water employed as wash liquid was extracted thoroughly with ethyl ether, and the ethyl ether was repeatedly washed with water before it was combined with the main ether fraction. The ether solution containing the saturated fatty acids, as well as the combined ethanol solution containing the unsaturated acids, was then evaporated and counted (Table V).

About equal amounts of C^{14} were recovered in the saturated and unsaturated fractions, whose weights were in the ratio 1:2. Approximately 10% of the solids and 25% of the radioactivity were not recovered. The saturated fatty acid which had been precipitated twice from ethanol showed a significantly higher specific activity than either the crude or unsaturated fractions.

DISCUSSION

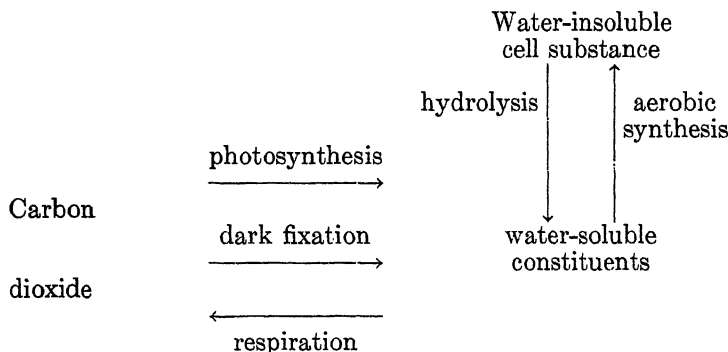
The present investigation has demonstrated that C^{14} enters several algal lipide fractions, and is already distributed among them rather uniformly, in terms of specific activity, during the first minute of exposure. The rapidity with which the tracer appears in these fractions, which are only remotely connected with photosynthesis, is not at variance with existing knowledge of enzyme working periods; some enzymes are known to have working periods as short as 1×10^{-5} sec. (20). Emerson and Arnold (21) observed a working time of *ca.* 0.02 sec. for the dark reaction which limits and controls the over-all rate of photosynthesis at 25°C., high CO_2 concentration, and high light intensity. These are also the conditions which have been employed in most of the reported tagging experiments. The intermittent light investigations indicate that the nonlimiting enzyme reactions connected with photosynthesis either are catalyzed by enzymes having a working period shorter than 0.02 sec. under the same conditions, or are catalyzed by enzymes present in much greater numbers than in the rate-limiting reaction.

It was previously indicated (1) that the tracer in water-soluble substance after brief photosynthetic exposures is almost entirely in a stable photosynthetic intermediate. This earlier conclusion is obviously difficult to reconcile with the present findings concerning the benzene-soluble fractions.

Examination of the earlier data (1) discloses details to which attention has not previously been directed. Figure 8 (Ref. 1, p. 420) shows an almost constant amount of photosynthesized tracer in water-solubles before and after 2 hr. of either aerobic or anaerobic dark metabolism; the amount of water-soluble tracer actually increased slightly during each of the 2-hr. dark treatments. The water-insoluble fraction contained more tracer than the water-solubles in this experiment. The data suggest that the insoluble cell substance contributed, by hydrolysis,

slightly more tracer to the water-soluble fraction than was simultaneously lost from it by respiration, exchange, and synthetic reactions. Figure 5 (Ref. 1, p. 417) shows that the tracer incorporated in the water-solubles by anaerobic dark fixation is quite rapidly lost by respiration, exchange, and synthetic reactions when the cells are maintained in darkness, in either the presence or absence of oxygen. In this experiment, the tracer was initially concentrated in the water-solubles, and losses from this fraction could not be compensated, as in the experiment with photosynthetically-incorporated tracer.

The dissimilar behavior of the tracer assimilated in light and in darkness during subsequent dark metabolism (1) is now attributed to the initially dissimilar distribution of tracer among the solvent fractions, and to the operation of the indicated cycle.



According to this interpretation, the previously observed differences in metabolic behavior of the light and dark fixation products could be abolished or even reversed by appropriate changes in the initial distribution of tracer among the solvent fractions.

Calvin and Benson's data concerning the very rapid tagging of many water-soluble constituents (22,23) are in essential agreement with the present observations on the benzene-soluble fraction—a very rapid and widespread distribution of the tracer carbon has now been observed in each of these algal fractions. During 30–60-sec. photosynthetic exposures to $C^{14}O_2$, algae apparently incorporate similar amounts of C^{14} in sucrose and in the lipide fractions (approximately 5% of the total assimilated in each case). Very short exposures are required for the appearance of C^{14} in the benzene-soluble fraction; as Brown, Fager,

and Gafron have also shown, the tracer reaches the algal colloids or "C" fraction during the first minute of exposure (1). In interpreting the early distribution of C^{14} among a large number of water-soluble compounds (22,23), it needs to be borne in mind that many compounds in addition to sucrose and sucrose precursors are being synthesized. Intermediates and products of many synthetic processes other than sucrose formation should rapidly become radioactive under tagging conditions which permit growth.

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SUMMARY

1. The tracer carbon found in the benzene-soluble fraction of *Scenedesmus obliquus* after 40-sec. exposures to $C^{14}O_2$ is incorporated in nonvolatile water-insoluble compounds. The radioactivity of the "A" fraction does not result from trace contamination with water-soluble compounds.

2. Chlorophylls *a* and *b* were obtained free of tracer carbon by successive purifications on sucrose columns. The chlorophyll was not separated from the tagged non-pigments on MgO and $MgO-Ca(OH)_2$ columns.

3. After saponification, the "A"-fraction tracer carbon was distributed quite uniformly, in terms of specific activity, between the unsaponifiables, fatty acid, and water-soluble saponification products. Very little of the tracer carbon in these fractions was removed by lengthy steam distillation. Approximately equal amounts of tracer were recovered in the saturated and unsaturated fatty acid fractions which were separated by the lead salt-alcohol method.

4. These indications of the rapidity of photosynthetic and other cell processes are discussed in relation to the known working periods of enzymes and the responses of photosynthesis to intermittent light. The different behavior of tracer assimilated in light and in darkness during subsequent dark metabolism (1) is attributed to the initial distributions of tracer carbon among the three solvent fractions.

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The Inhibition of Chicken-Bone-Marrow Choline Oxidase By Aminopterin *in Vivo*¹

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INTRODUCTION

We have previously reported (1) that liver and kidney tissue from aminopterin-treated monkeys exhibited very little choline-oxidase activity as compared with such tissue from control monkeys. Barron *et al.* (2) have reported that the nitrogen mustards strongly inhibit rat-liver choline oxidase *in vitro*, and we have found that urethan also inhibits this enzyme. Since these three substances (aminopterin, nitrogen mustard, urethan) also induce leucopenia in animals, it seemed possible that their effects on blood cell formation were related to their effect on choline oxidase. A study has been made of the respiration of bone marrow from control and aminopterin-treated chickens, and of the effects of addition of choline chloride and sodium succinate as substrates to the marrow preparations. The results indicate that normal chicken-bone marrow exhibits choline-oxidase activity and that aminopterin treatment completely inhibits this bone-marrow enzyme.

EXPERIMENTAL

Adult hens were injected with 1 mg. of aminopterin daily for periods of from 2 to 13 days. Uninjected hens served as controls. Complete blood counts were made on blood taken from the wing veins; the hens were killed by decapitation and the tibias and femurs dissected free of muscle tissue and placed in an ice bath. The bones were cracked by gentle tapping and the marrow was removed as completely as possible.

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All of the marrow thus obtained was stirred with 0.9% NaCl solution at 0°C., strained through gauze, and made up to an appropriate volume, usually 10 ml. Marrow cell counts were made on an aliquot of this suspension using a red blood cell diluting pipet and a blood cell counting chamber. In other experiments the chilled marrow was homogenized with 0.05 *M* phosphate buffer at pH 7.8 in a Potter-Elvehjem apparatus. A control was run with each experimental bird. Oxygen consumption was measured for 3 hr. by standard Warburg procedures. The final volume in the flasks was 3.2 ml. containing 1 ml. of the marrow preparation and 1 ml. of 0.05 *M* phosphate buffer at pH 7.8 Five mg. of choline chloride or 5 mg. of sodium succinate was added as substrate to certain flasks.

RESULTS

The effects of aminopterin on marrow cell counts and on peripheral blood cell counts are shown in Table I. It may be seen that the marrow cell counts and peripheral leucocyte counts were rapidly reduced by aminopterin. A longer period of treatment was necessary to significantly reduce blood erythrocyte counts.

TABLE I

The Effects of Aminopterin Treatment on Chicken-Bone-Marrow Cell Counts and on Peripheral Blood Cell Counts

| Treatment duration | Cell counts as per cent of normal | | |
|--------------------|-----------------------------------|------------------|-----------------|
| | Marrow cells | Peripheral blood | |
| | | Leucocytes | Erythrocytes |
| <i>days</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| 4 | 55 | 65 | 100 |
| 5 | 18 | 36 | 92 |
| 6 | 11 | 28 | 63 |
| 13 | 15 | 30 | 53 |

The effect of choline on the oxygen consumption of normal chicken-marrow cell suspensions is shown in Fig. 1. Choline markedly stimulated oxygen uptake and the rate was essentially constant over the 3-hr. period.

The effect of aminopterin treatment on the choline-oxidase activities of marrow cell suspensions is shown in Table II. It may be noted that bone-marrow cell suspensions from control chickens exhibited marked choline-oxidase activity. The addition of choline to these cell suspensions increased oxygen consumption from 33 to 71% (average 46%).

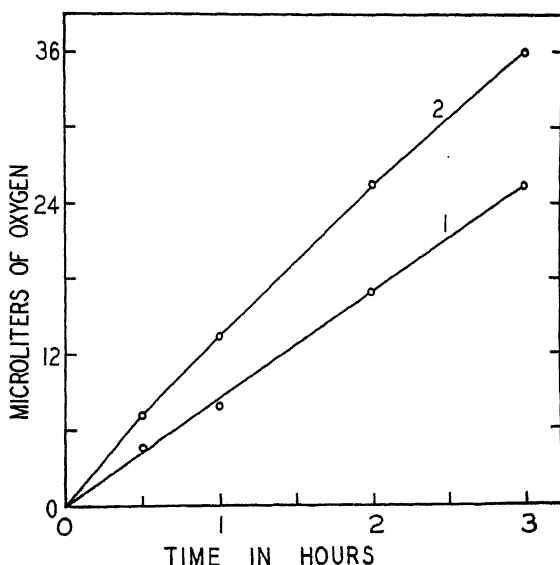


FIG 1. Effect of choline on the oxygen consumption of normal chicken-bone-marrow cell suspension. Curve 1, cell suspension; curve 2, cell suspension plus choline chloride (0.011 *M*). Oxygen consumption in microliters of oxygen/mg. of nitrogen.

Marrow cell suspensions from aminopterin-treated chickens did not exhibit choline oxidase and after 5 days of aminopterin treatment the addition of choline to the marrow cell suspensions depressed oxygen consumption. The choline-oxidase activity of bone-marrow homoge-

TABLE II

The Effects of Aminopterin Treatment on the Choline-Oxidase Activity of Chicken-Bone-Marrow-Cell Suspensions

| Duration of treatment | | Effect of addition of choline on oxygen consumption ^a |
|-----------------------|------------------|--|
| days | | per cent |
| 0 (controls) | (7) ^b | + 46 |
| 2 | (2) ^b | + 6 |
| 4 | (2) ^b | + 2 |
| 5 | (1) ^b | - 18 |
| 6 | (1) ^b | - 29 |
| 12 | (1) ^b | - 27 |

^a The change in oxygen consumption relative to the endogenous uptake.

^b Number of chickens.

nates is illustrated in Table III. The addition of choline to marrow homogenates from control chickens resulted in a marked increase in oxygen consumption while the addition of choline to homogenates from aminopterin-treated chickens depressed oxygen consumption.

Aminopterin treatment did not affect the succinic oxidase activity of chicken-bone-marrow cell suspensions in these experiments; the addition of sodium succinate to marrow cell suspensions from control and aminopterin-treated chickens increased oxygen consumption 22% and 21%, respectively.

TABLE III

The Effect of Aminopterin Treatment on the Choline-Oxidase Activity of Chicken-Bone-Marrow Homogenates

| | Oxygen consumption/mg. N/hr. | |
|------------------|-------------------------------|---|
| | Control $\mu\text{l. O}_2$ | Aminopterin-treated $\mu\text{l. O}_2$ |
| Marrow | 9.8 | 8.1 |
| Marrow + choline | 18.0 | 6.1 |

In contrast to the marked effect of aminopterin treatment on chicken-bone-marrow choline oxidase was the mild effect on kidney choline oxidase. Aminopterin treatment reduced kidney choline oxidase from 18 to 26% with an average reduction of 22%. Chicken-liver choline oxidase was not reduced by aminopterin treatment under the condition of these experiments. These observations may indicate a predilection of aminopterin for bone marrow, or they may indicate that chicken-bone-marrow choline oxidase is a different enzyme from chicken liver and kidney choline oxidase.

DISCUSSION

Goldinger *et al.* (3) reported that rabbit bone marrow did not contain choline oxidase and their observation has been confirmed in this laboratory. Since the effects of aminopterin on blood-cell formation seems to be related to its effect on choline oxidase, we studied the effects of aminopterin treatment on the rabbit. In one experiment, as yet unpublished (4), an adult albino rabbit weighing approximately 4 kg. was injected intramuscularly with a total quantity of 665 mg. of aminopterin over a 4-month period; during the last month of that period the animal received 12.8 mg. daily. There was no effect either on the general appearance of the rabbit or on its hemogram. Since the daily injection of 0.8 mg. of aminopterin daily for 7 days may prove fatal to a 3 or 4

kg. monkey, the resistance of this rabbit was quite surprising. It may be related to the absence of choline oxidase in rabbit bone marrow.

The experiments reported in this paper demonstrate that the reduction in chicken-bone-marrow cell counts and peripheral leucocyte counts by aminopterin treatment is accompanied by a loss of bone-marrow choline oxidase. The observations that rabbit bone marrow does not contain choline oxidase and that aminopterin does not produce a leucopenia in this species strengthen the possibility that the leucopenic effect of aminopterin is related to its effect on choline oxidase. The nitrogen mustards and urethan act on choline oxidase *in vitro*. On the contrary, aminopterin is effective in reducing choline oxidase *in vivo* but not *in vitro*. This may suggest that aminopterin acts by displacing folic acid from a position in the choline-oxidase enzyme system. Other choline-oxidase inhibitors are being studied to determine their effects on leucocyte formation.

SUMMARY

A study was made of the effects of aminopterin treatment on the peripheral blood cell counts, bone-marrow cell counts, and bone-marrow choline oxidase activity of adult chickens. The daily injection of 1 mg. of aminopterin reduced peripheral leucocytes and total marrow cells to about 50% of control values in 4 days; peripheral erythrocytes were reduced to 53% of control value after 13 days of aminopterin treatment. Bone marrow from control chickens exhibited choline oxidase activity while marrow from aminopterin-treated chickens did not exhibit choline oxidase. It is suggested that aminopterin inhibits bone-marrow choline oxidase by displacement of folic acid from a position in the enzyme system.

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Inhibitors for Penicillinase

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INTRODUCTION

One of the mechanisms by which bacteria overcome or prevent the inhibitory action of penicillin involves the enzyme penicillinase.¹ This enzyme destroys penicillin, opening the β -lactam ring to form penicilloic acid.² Inhibitors for penicillinase may be capable of extending the antibiotic action of penicillin to some organisms or strains not susceptible to the antibiotic alone. Housewright and Henry³ have investigated the possible use of antipenicillinase serum for this purpose. Because of the impermeability of the cell walls to the immune proteins, these workers concluded that sensitivity to penicillin could be increased only in those organisms which produced extracellular penicillinase. Since it appears probable that penicillinase is an intracellular product which is retained by some bacteria and is released to some extent by other bacteria, smaller molecules acting as penicillinase inhibitors and capable of penetrating cells may be more effective than antipenicillinase. Compounds related in structure to a portion of the penicillin molecule appeared to offer good possibilities for this purpose inasmuch as some of the groupings of the penicillin molecule are undoubtedly of importance in making possible the combination with the enzyme. However, very little information is available to indicate which portions of the structure are important. Previous work in this laboratory⁴ has indicated that the acyl portion of the penicillin molecule is not highly specific in determining which compounds can combine with penicillinase, for all of the biosynthetic penicillins tested were readily destroyed by this enzyme. Comparable data for other portions of the molecule are not available. In a search for penicillinase inhibitors, it was necessary, therefore, to test a variety of structures. The data presented below show that a number of compounds inhibit penicillinase,

and furthermore, several of these compounds markedly sensitize a penicillinase-forming organism to penicillin.

Reid, Felton, and Pittroff ⁵ had shown previously that several structures, not obviously related to penicillin, inhibited destruction of penicillin by a crude penicillinase preparation (Clarase). These authors also reported that one of these compounds, sodium sulfanilate, potentiated the action of penicillin in mice infected with *Diplococcus pneumoniae*, Type I. The manner of action of sodium sulfanilate in this experiment appears to be uncertain. Probably this organism does not produce appreciable penicillinase. Furthermore, separate experiments in uninfected rabbits indicated that administration of sodium sulfanilate caused a prolongation of penicillin blood levels.

EXPERIMENTAL

The effect of various compounds on the penicillinase-penicillin reaction was determined manometrically in constant-volume Warburg respirometers in a manner similar to that previously described.⁴ The substance under test as a penicillinase inhibitor was dissolved in the bicarbonate buffer. Acidic substances were first neutralized. Each vessel contained 1×10^{-5} moles of sodium benzylpenicillin (2.56 mg.) in a total volume of 3.5 ml. A purified penicillinase (Penicillinase A, Schenley Laboratories) was used in all experiments. The test substances were added in quantities up to 1×10^{-4} moles/flask except in instances in which lack of solubility prevented use of this concentration. The following compounds failed to show significant inhibition in concentrations up to 1×10^{-4} moles/vessel: phenaceturic acid, benzylpenicilloic acid, *N*-phenylacetyl-glycyl-DL-valine, *N*-2-aminoethylphenylacetamide hydrochloride, DL-valine, DL-homocysteine, and 2-benzylimidazole-4,5-dicarboxylic acid. The following compounds failed to show significant inhibition with quantities up to 1×10^{-5} moles/flask: *N*-phenylacetyl-DL-alanyl-DL-valine, *N,N'*-diphenylacetyl-L-cystinyl-diglycine, *N*-phenylacetyl-glycyl-DL-penicillamine, *N*-phenylacetyl-DL-seryl-DL-valine, *N*-phenylacetyl-DL-penicillamine, 2,2,5,5-tetramethyl-3-phenylacetylthiazolidine-4-carboxylic acid, 3-carboxy-2-phenylthiazolidine, 5,5-dimethyl-2-phenylthiazolidine-4-carboxylic acid, 2-(*o*-chlorophenyl)-4-carboxythiazolidine, 3-phenylpyrazole, 1-benzylpyrazole, 1-benzylimidazole, 2-benzyl-4,5-dihydroimidazole, 1,3-dimethyl-2-hydroxy-2-phenylbenzimidazole, 2-benzylthiazole-4-carboxylic acid, *N*-acetyl-*N*-phe-

nylglycine, and 2-imidazolylalanine. In addition, no effect was observed in the presence of 1×10^{-6} moles of the benzylamide of penicilloic acid.

Table I lists a number of compounds which have caused significant inhibition of penicillinase. It is of interest to compare the inhibition observed with these compounds with that caused by benzoic acid.⁵ Under our conditions benzoic acid gave an inhibition of 0.24 when 3×10^{-3} moles were added to each vessel, 0.75 with 1×10^{-3} moles, and 0.87 with 5×10^{-4} moles. It is apparent that benzoic acid is a weaker inhibitor than any of the compounds listed in Table I.

TABLE I

Inhibitors for Penicillinase

The values represent the ratio: rate of CO_2 evolution in the presence of the compound/rate of CO_2 evolution of control.

| Compound | Amount of compound added (moles) | | | |
|--|----------------------------------|--------------------|--------------------|--------------------|
| | 1×10^{-4} | 1×10^{-5} | 1×10^{-6} | 1×10^{-7} |
| 2-Methylimidazole | 0.37 | 0.64 | 1.0 | 0.82 |
| 2-Benzylimidazole | | 0.36 | 0.74 | |
| 2-Aminomethylthiazole | 0.30 | 0.80 | 1.0 | |
| L-Penicillamine | 0.62 | 0.92 | 1.0 | |
| D-Penicillamine | 0.58 | 0.90 | 1.0 | |
| DL-Penicillamine | 0.59 | 0.89 | 1.0 | |
| DL-Thiothreonine | 0.29 | 0.94 | 1.0 | |
| DL-Cysteine hydrochloride | ? | 1.0 | 1.0 | |
| L-Cysteine hydrochloride | ? | 1.0 | 1.0 | |
| Histamine | 0.10 | 0.84 | 1.0 | |
| p-Chlorobenzoyl-DL-seryl-DL-valine | | 0.68 | 1.0 | |
| 2-Propyl-4-thiazolidinecarboxylic acid | 0.08 | 1.0 | 1.0 | |
| Benzylpenicillic acid | 0.34 | 0.74 | 1.0 | |

Each of the α -amino- β -mercapto compounds that was tested (penicillamine, thiothreonine, and cysteine) acted as an inhibitor. Definite values for the inhibition with L- and DL-cysteine cannot be given as an unexplained preliminary loss of gas was noted in most of the experiments in which 1×10^{-4} moles of these compounds were used. The loss was of such magnitude that we expected to observe it in the flasks containing 1×10^{-5} moles of cysteine, if it were directly related to the

cysteine content. However, no evidence of gas loss was noted at the lower concentrations.

The lack of optical specificity observed with L- and D- penicillamine seems surprising. It contrasts with the specificity observed in the organic synthesis of penicillin in which only the D-isomer of penicillamine leads to a biologically active product.⁶ Similarly, the toxicity of penicillamine for rats is a property of the L-isomer and is not shared by the D-anti-pode.⁷

Experiments were performed to determine whether penicillinase inhibitors would increase the susceptibility of a penicillinase-forming organism to penicillin. *Bacillus cereus* NRRL B-569, a good producer of penicillinase, was used in these tests. An inoculum of 0.03 ml. of

TABLE II

Growth of Bacillus cereus in the Presence of Varying Concentrations of Benzylpenicillin and of Various Penicillinase-Inhibiting Compounds

| Compound added (3×10^{-6} M/ml. | Penicillin, u.g./ml.; Inoculum, 0.03 ml. undiluted | | | | | Penicillin, u.g./ml.; Inoculum, 0.03 ml. 1:100 | | | | | |
|--|--|-----|-----|----|---|--|-----|-----|----|----|---|
| | 1500 | 500 | 150 | 50 | 0 | 1500 | 500 | 150 | 50 | 15 | 0 |
| None | — | + | + | + | + | — | + | + | + | + | + |
| D-Penicillamine hydrochloride | | — | — | — | + | | — | — | — | + | + |
| D,L-Penicillamine hydrochloride | | — | — | + | + | | — | — | — | — | + |
| 2-Benzylimidazole | | — | + | + | + | | — | ± | + | + | + |
| 2-Methylimidazole | | ± | + | + | + | | + | + | + | + | + |
| 2-Aminomethylthiazole | | ± | + | + | + | | + | + | + | + | + |

24-hr. culture (either undiluted or diluted 1:100) was added to 4 ml. of nutrient broth containing the desired concentration of penicillin. One ml. of a solution of the penicillinase-inhibitor compounds, which had previously been neutralized and sterilized by autoclaving, was then added. The tubes were incubated at 37°C. for 24 hr. Table II lists the results of these experiments. Both D- and D,L-penicillamine markedly enhanced the effect of penicillin. In the absence of these compounds approximately 10–30 times more penicillin was required to inhibit growth of the organism than when either of the compounds was present. In contrast, 2-benzylimidazole, 2-methylimidazole, and 2-aminomethylthiazole, compounds which were more active than penicillamine in the

antipenicillinase tests, produced little or no enhancement of penicillin activity in the tests with *B. cereus*.

ACKNOWLEDGMENTS

The compounds *N*-phenylacetyl-glycyl-DL-valine and *N*-phenylacetyl-glycyl-DL-penicillamine were supplied through the courtesy of The Abbott Research Laboratories; the benzylamide of penicilloic acid was supplied by The Upjohn Company; and DL-thiolthreonine was furnished by Dr. H. E. Carter.

CONCLUSION

Tests for antipenicillinase activity have been performed with a number of compounds more or less structurally related to a portion of the penicillin molecule. Several have exhibited marked antipenicillinase activity. These included 2-benzylimidazole, 2-methylimidazole, benzylpenicillic acid, 2-aminomethylthiazole, *p*-chlorobenzoyl-DL-seryl-DL-valine, histamine, 2-propyl-4-thiazolidinecarboxylic acid and the α -amino- β -mercapto compounds, penicillamine, thiolthreonine, and cysteine.

Several of these compounds were tested for their ability to enhance the effectiveness of penicillin on a penicillinase-producing organism, *Bacillus cereus*. The effectiveness of the substances in this test did not parallel that in the antipenicillinase test. Penicillamine markedly potentiated the activity of penicillin, but 2-benzylimidazole, 2-methylimidazole, and 2-aminomethylthiazole had little or no effect.

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The Significance of the Fractionation of Hemicelluloses of Cornstalks¹

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INTRODUCTION

Angell and Norris (1) have indicated that a hemicellulose fraction does not represent a definite chemical entity and that hemicelluloses of the same designation but prepared from different plant sources are not necessarily identical. Hence, systematic designations indicate not definite classes of hemicelluloses but particular methods of preparation. Preece (2) has also indicated that none of the methods in use achieves a rigid differentiation between encrusting hemicelluloses and cellulosans. It becomes rather obvious that fibrous material from many sources is so heterogenous, in that it is composed of intermeshing systems, that a separation of constituents is likely to be empirical at best unless conditions for removal are standardized.

The investigation to be presented deals with the successive extraction of hemicelluloses from holocellulose of cornstalks by four solvents of different strengths. Four different fractions were thus obtained which were perhaps increasingly difficult to extract. The data obtained tend to indicate that it is possible to obtain fractions which in many respects are significantly different.

EXPERIMENTAL

The entire aerial portion of the mature stalk (*Zea mays*) was ground to pass a 50-mesh but be retained by a 100-mesh sieve. The following methods of analysis were used: Holocellulose was determined essentially by the method of Wise *et al.* (3,4). Extractions were made with water, 2% sodium carbonate, 4% sodium hydroxide, and 10% sodium hydroxide; the extracts will be known hereafter as A, B, C, and D respectively.

¹ Contribution No. 753, Massachusetts Agricultural Experiment Station.

The content of pentosans and methoxyl was determined by methods of the A. O. A. C. (5). Optical activity was measured in a Haensch and Schmidt saccharimeter. Periodate oxidations were conducted essentially as described by Brown and associates (6), except that potassium chloride was not used and the temperature was maintained at 15–17°C. (4).

Evidence in Table I indicates that certain variations do occur which are associated with resistance to extraction. In this case it would seem that the purer the pentosan, the more difficult it is to extract. On the basis of solubility alone, one might expect that the least soluble fraction would contain the longer polymers. Since such variations might be shown by very small fractions, the percentage distribution of each fraction is also included in the table. The decidedly strong negative optical activity is characteristic of all fractions and indicates that the residues making up the polymers are of the beta configuration. The moles of periodate consumed per sugar unit indicate that fractions *B*, *C*, and *D* probably have the same glycosidic linkage. If the pyranose structure is predominant, the 1,2- or 1,4- glycosidic linkage for the anhydropentososes is the only linkage providing vicinal hydroxyls, and hence is the only arrangement which allows for the consumption of one mole of periodate per sugar residue. Failure to consume this amount of periodate, as evidenced in fraction *A*, might indicate the following: (a) Impurities may be present; (b) one of the vicinal hydroxyl groups present in either a 1,2- or 1,4- glycosidic linkage might be combined with another body; or (c) a 1,3- glycosidic linkage might be present.

The average number of sugar residues per nonreducing end group of each fraction as recorded in Table II also tends to substantiate the

TABLE I

The Specific Rotation and Percentage Content of Pentosans, Methoxyl and Uronic Acid Anhydride in Each Fraction of Total Hemicelluloses From Cornstalks

| Fraction | Specific rotation | Pentosans | Methoxyl | Uronic acid anhydrides | Percentage of total hemicellulose |
|----------|-------------------|-----------|----------|------------------------|-----------------------------------|
| | $[\alpha]_D^{20}$ | % | % | % | % |
| <i>A</i> | –56.6 | 67.0 | 1.63 | 7.73 | 8.0 |
| <i>B</i> | –78.8 | 88.0 | 1.14 | 11.07 | 27.1 |
| <i>C</i> | –101.2 | 98.7 | 0.97 | 4.39 | 50.2 |
| <i>D</i> | –101.4 | 98.5 | 0.63 | 3.06 | 14.8 |

TABLE II

The Moles of Periodate Consumed Per Sugar Residue and the Average Number of Sugar Residues Per Nonreducing End Group Present in the Hemicellulose Fractions

| Fraction | Moles periodate consumed per sugar residue | Weight of sample | Net formic acid | Av. no. sugar residues per non-reducing end group |
|----------|--|------------------|-----------------|---|
| | | <i>mg.</i> | <i>mg.</i> | |
| A | 0.80 | 264.5 | 5.25 | 17 |
| B | 0.93 | 259.5 | 7.90 | 11 |
| C | 0.95 | 260.6 | 3.70 | 24 |
| D | 1.00 | 262.4 | 2.95 | 30 |

findings in Table I. Again, with the exception of fraction A, the relative size of the numbers indicating the average number of sugar residues per nonreducing end group is what one might expect on the basis of solubility alone. However, when one considers the heterogeneity of the intermeshing systems present in the cell wall, it is somewhat surprising that so close a parallel in apparent chain length should exist between what one might expect on the basis of solubility alone and what was actually found.

SUMMARY

In general the fraction most difficult to extract is characterized by the lowest percentage content of methoxyl and uronic acid anhydrides, but the highest percentage content of pentosans, the greatest number of sugar residues per nonreducing end group, and consequently, the highest apparent molecular weight, and is, therefore, considered significantly different. The sugars seem to be linked by either the 1,2- and/or 1,4- glycosidic linkage, in which the beta configuration predominates.

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Determination of Adenosine Triphosphate Based on Deamination Rates¹

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INTRODUCTION

This paper describes a spectrophotometric assay for adenosine triphosphate which may be applied either to unknown solutions or to tissue extracts. The method is an extension of a series of techniques developed by Kalckar (1,2). When combined with Kalckar's procedures, the method can be used for the simultaneous determination of adenylic acid (AA), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) at the micro level. In principle the method consists of converting adenosine triphosphate enzymatically into adenylic acid with a combined hexokinase-myokinase system and measuring the rate of inosinic acid formation with a muscle deaminase in the spectrophotometer.

METHODS

Preparation of Enzymes

Adenylic acid deaminase (Schmidt's deaminase) was prepared according to the method of Kalckar (3); myokinase, according to Colowick and Kalckar (4). Hexokinase was prepared in partially pure form according to the method of Berger, Slein, Colowick, and Cori (5) through step 3 (fractionation with ethanol at 0°C.) of the isolation procedure. (See *Experimental*.) The enzymes were kept in deep freeze and were found to be stable over long periods of time.

EXPERIMENTAL

The determination was carried out in the following way: Varying amounts of ATP (Ernst Bischoff Company, Ivoryton, Connecticut) were used, 0.5 ml. 0.1 *M* malonate buffer pH 5.9, 0.1 ml. 0.5 *M* glucose, 0.1 ml. MgCl₂ (10 mg./ml.), 0.01 ml. myokinase

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(containing 4 μ g. protein N), 0.01 ml. hexokinase (containing 7 μ g. protein N) water to 4.9 ml., and 0.03 ml. of the deaminase added last (containing 15 μ g. protein N).

In early experiments the change in density was recorded every 2 min. after the addition of the deaminase for varying lengths of time (Fig. 1). It is apparent that the course of the reaction is linear for at least the first 10 min. Plotting ATP (expressed as micrograms of adenine) against 10-min. change in density at 265 $m\mu$ gives a straight line (Fig. 2, \bullet).

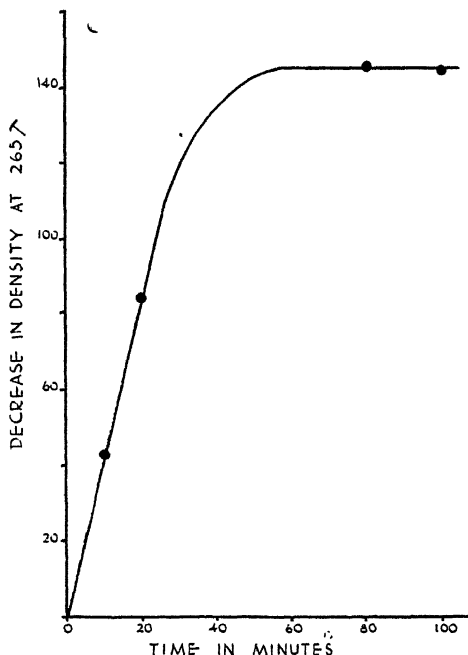


FIG. 1. Time course of deamination of adenylic acid formed from adenosine triphosphate in presence of complete hexokinase-myokinase system. See text for experimental details.

In assay procedure, 10-min. readings are used and levels of ATP are read directly from the curve.

In the above series of reactions it is necessary to scale the concentrations of myokinase and hexokinase in such a way that the adenylic acid is formed more rapidly than it is deaminated by the deaminase. If this is done then the method can also be used for the assay of adenylic

acid, and adenosine diphosphate as well as ATP. In such a case the curve for all three compounds plotted on a micrograms adenine basis should be identical (see Fig. 2 ○, and ●). In applying these methods, therefore, preliminary experiments should be carried out to determine nonlimiting levels of hexokinase and myokinase. In our experience some preparations of hexokinase gave low activity. To insure complete conversion of ATP to ADP, all the ingredients were mixed together with the exception of the deaminase which was added after a time interval of about 30 min. In this connection, the same result could have been achieved in a shorter time interval by raising the level of hexokinase.

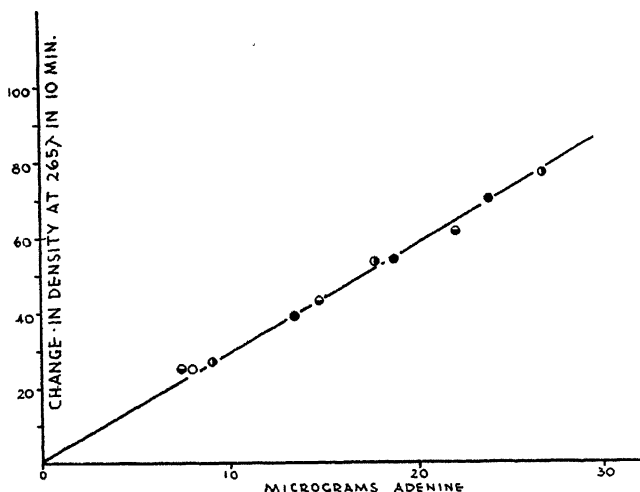


Fig. 2. Relationship between 10-min. density change at 265 $m\mu$ and concentration of adenine (micrograms) in various adenine nucleotides treated with different enzyme combinations: ○, adenylic acid; ○, adenosine diphosphate; ●, adenosine triphosphate; ●, coenzyme I.

This was not done because it was desirable to keep the density due to the enzymes themselves at a minimum.

Measurement of Adenylic Acid, Adenosine Diphosphate and Adenosine Triphosphate in Unknown Solutions

Since all three compounds give identical results when concentration, expressed as micrograms of adenine, is plotted against density, it should be possible by using different combinations of enzymes to determine all

three constituents in an unknown solution. This has been done in assaying four different preparations of adenosine triphosphate. Two were barium salts (*A* and *B*), one was a sodium salt (*C*), and the fourth was the free acid (*D*), the label of the latter claiming a purity of 40%. The barium salts were freed of barium and all were assayed chemically by measuring the adenine, pentose, labile, and total phosphorus. The results of the chemical analyses are shown in Table I. The samples were then assayed spectrophotometrically in the following way: Equal aliquots of each compound were placed in 3 separate tubes. Into the first tube was pipetted water, buffer, and deaminase and the change in density at 265 $m\mu$ at the end of 10 min. was recorded. The second tube received buffer, $MgCl_2$, water, myokinase, and deaminase and again the change in density was recorded at the end of 10 min. To the third

TABLE I

Purity of Various Commercial Preparations of Adenosine Triphosphate

| Preparation | Molar ratio | | | | Purity calculated from labile P | Purity calculated as ATP spectrophotometrically | Per cent of total purine as | |
|-------------|-------------|---------|----------|---------|---------------------------------|---|-----------------------------|------|
| | Adenine | Pentose | Labile P | Total P | | | AA | ADP |
| <i>A</i> | 1.04 | 1.04 | 2.00 | 3.08 | % 96 | % 98 | 0 | 0 |
| <i>B</i> | 0.81 | 0.82 | 2.00 | 2.88 | 104 | 64 | 0 | 13.3 |
| <i>C</i> | 1.02 | 0.99 | 2.00 | 2.99 | 88 | 89 | 0 | 0 |
| <i>D</i> | 3.25 | | 2.00 | 5.81 | 38 | 13 | 72.6 | 0 |

tube was added the complete system as outlined above for the assay of ATP. Any change which occurs in the first tube is due to adenylic acid alone. Any change in tube 2 over that occurring in tube 1 is due to the presence of ADP. Since only 1 mole of AA is formed from 2 moles of ADP, the difference between tubes 1 and 2 is multiplied by a factor of two. The third tube records the change in density due to all three compounds. Since the concentrations of AA and ADP are now known (by reading the adenine due to these compounds from the curve), and the total adenine is known from tube 3, the difference between the AA and ADP adenine and the total adenine gives the value for the ATP adenine. The data, also presented in Table I, show that Prepn. *A* is the purest; it contains no AA and no ADP; Prepn. *B* contains no AA, but has small quantities of ADP; Prepn. *C*, like *A*, has neither AA nor ADP,

while Prepn. *D* contains large quantities of AA and relatively little ATP. It is interesting to point out that the purities of the different preparations in terms of their ATP content bear a relationship to the purity as determined from the labile phosphorus only when the compound is pure. If either ADP, AA, or other phosphorous compounds are present, then the labile P is a poor criterion of purity (see Prepn. *B* and *D*).

Assay for Levels of AA, ADP, and ATP in Blood

The content of these constituents in blood may be readily determined by drawing blood in an oxalated syringe and deproteinizing as quickly as possible with 4 volumes of 2% perchloric acid, as suggested by Kalckar (2). The precipitated protein, after centrifugation, is washed with another volume of 2% perchloric acid in the centrifuge. The super-

TABLE II
Nucleotide Content of Blood

| | Total purine expressed as adenine | Adenine as AA | Adenine as ADP | Adenine as ATP |
|---------------|---|---------------|----------------|----------------|
| | <i>mg.—%</i> | <i>mg.—%</i> | <i>mg.—%</i> | <i>mg.—%</i> |
| Frog blood | 19.3 | 0 | 0 | 13.9 |
| Cells | 29.0 | 0 | 0 | 23.5 |
| Plasma | 0.0 | | | |
| Chicken blood | 21.0 | 0 | 0 | 7.5 |

natant fluids are combined and carefully neutralized with 2 *N* NaOH and made to volume. One ml. of blood is made to a final volume of 10 ml., and 1-ml. aliquots of this neutralized supernatant are used for analysis as outlined above for the commercial preparations of ATP. Standard curves for AA, ADP, and ATP must be run in the presence of perchloric acid, since the presence of even neutralized perchloric acid affects the reaction rates. If the perchloric acid level is kept at or below the level described above, added nucleotides may be detected quantitatively. Higher concentrations of perchloric acid, as pointed out by Kalckar (2), inhibit the activity of the enzymes. The analyses shown in Table II were carried out on frog and chicken blood and are presented merely to show the applicability of the method. Neither blood con-

tained AA or ADP, but both contained ATP. The ATP appears to be present only in the red cells and not in the plasma. Similar results have been obtained with the blood of man. Extensive assays on human blood are now being carried out using the methods outlined. These will be described elsewhere.²

DISCUSSION

The method described above has several advantages over that initially described by Kalckar (2). First, it differentiates between adenosine diphosphate and adenosine triphosphate and enables one to assay for each separately in unknown solutions. Kalckar used a potato pyrophosphatase for his analysis of tissue extracts. Second, the determinations, since they are based on rate measurements, can be completed within a short time, *e.g.*, less than 15 min., as compared to the 3 hr. in the case which Kalckar describes.

Rate measurements have their disadvantages, especially when impurities which inhibit the test enzymes are present in unknown extracts. These can, however, easily be detected by adding known solutions to the unknowns. In some cases, for example, as in the assay of commercial preparations of adenosine triphosphate, inhibitions in rate of reaction may be of distinct advantage, since one is able to detect trace impurities. We have detected trace metal impurities in several preparations of ATP in this way.

SUMMARY

A method is described for the spectrophotometric assay of adenosine triphosphate (ATP) using enzymes. When the method is combined with procedures described by Kalckar, it can be used for the simultaneous assay of adenylic acid and adenosine diphosphate as well as

² When this work was completed, a nucleotide pyrophosphatase prepared by Dr. Kornberg of the National Institute of Health was made available to one of the authors (HGA). This enzyme acts on the pyrophosphate linkages of ATP and ADP as well as on coenzyme I, forming adenylic acid as an end product. If in the assay described above, a fourth tube is set up containing the complete hexokinase-myokinase system as well as the nucleotide pyrophosphatase, the method becomes applicable to the measurement of coenzyme I. Values for coenzyme I have been included in Fig. 2 (●).

adenosine triphosphate at the micro level. The method has been applied to the assay of various commercial preparations of ATP as well as to blood.

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The Essential Groups of Lysozyme, With Particular Reference to Its Reaction With Iodine

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INTRODUCTION

Lysozyme is a protein component of egg white, characterized by its ability to lyse or kill a variety of microorganisms, possibly through enzymatic attack on the mucopolysaccharides of the bacterial wall. It is of widespread occurrence also in mammals, though present nowhere in similar concentration as in egg white (1-4). Because of the ease of isolation (5) and the stability of this well-defined protein of comparatively low molecular weight, it appeared well suited for a study, by chemical modification, of the mechanism of its enzymatic action. A similar study has recently been carried out on the effects of chemical agents on another egg white protein, ovomucoid (6), characterized by its ability to combine with and thereby inhibit trypsin (7). The latter two proteins, like other enzymes, hormones, and viruses previously studied [reviewed in Refs. (8, 9)] were found to retain their specific activities after various chemically reactive groups had been extensively modified. In contrast, the present investigation has shown the lytic activity of lysozyme to be very sensitive to most chemical agents. It is concluded that numerous types of reactive groups are involved in the "active site" of this enzyme.

The reaction with iodine was studied in some detail, because it leads to partial inactivation, which is reversible by means of reduction. It appeared that the single histidine residue of lysozyme is responsible for this phenomenon. In lysozyme, this amino acid is more reactive than in most proteins, while the tyrosine residues are less reactive.

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METHODS AND MATERIALS

The lysozyme was prepared by direct crystallization according to Alderton *et al.* (5). Most of the methods of chemical modification were the same as used in previous publications (6, 9, 10). The finding of Meyer *et al.* (3), confirmed by Alderton *et al.* (11), that lysozyme could be partially inactivated and subsequently reactivated by treatment with iodine and sulfite, led to a thorough study of this sequence of reactions when applied to lysozyme and other proteins. The conditions of *iodination* used in most of these experiments were as follows: 100 mg. of protein dissolved or suspended in a mixture of 6 ml. water, 0.2 ml. 0.1 *N* sodium hydroxide, and 0.3 ml. of 3.4 *M* phosphate buffer having a pH of 7.6 or 6.2, was treated with 3.5 ml. 0.1 *N* iodine (KI_3) solution. After 10 or 20 min. at room temperature, aliquots of the brown suspension were treated with just enough 0.1 *N* sodium sulfite or thioglycol to terminate the reaction by reduction of the free iodine. Parts of the almost white reaction mixtures were then treated with again as much of the various reducing agents, and all were dialyzed after another 10 min.²

Iodination of the phenolic groups was also attempted by treating the protein solutions, buffered with 0.3 *M* acetate at pH 5.2, with 0.1 *N* iodine solution, either in excess or 0.1 ml. at a time whenever the yellow color had almost completely disappeared. Very little iodine was bound by lysozyme at this pH and some oxidation occurred.

For the *oxidation* of the disulfide and other groups, performic acid was used, as well as periodate (12). To avoid precipitation the protein was treated in rather dilute solution (1.25%) with 1% sodium periodate in 0.2 *M* pH 5.5 acetate. The periodate concentration was determined after various intervals by addition of 2 drops of ethylene glycol to 2.0-ml. aliquots of the reaction mixture, and determination of the amount of formaldehyde formed in these test samples after 1 to 2 min. by means of dimedon (13). To correct for small amounts of protein which may precipitate together with the dimedon-formaldehyde condensation product, the latter was washed out with acetone and the protein weighed back. The reaction was also performed in 8 *M* urea solution (3.3% lysozyme) for 30 or 60 min. In all experiments, the oxidation of disulfide bonds was incomplete.

The isolation of lysozyme derivatives by *dialysis* represented special problems because of the ability of this protein to pass through most sizes of the Visking sausage casing used routinely for dialysis. Only spools of 2.3-mm. (dry, folded) diameter were found to retain about 80% of the protein after dialysis for 24 hr. against running tap water at 20°, and 4 days against daily changes of distilled water at 3°. The dialyzability of lysozyme (mol. wt. about 15,000) was unexpected in comparison with other materials of similar molecular weight. Even the lima bean trypsin inhib-

² Attempts to terminate the iodination by saturation of the reaction mixture with ammonium sulfate were unsuccessful. Almost all the iodine precipitated with the protein, and iodination continued to proceed during dialysis. Thiosulfate could be used to terminate the iodination but the reversal of the reaction was not possible with this reagent, nor in the presence of oxidized thiosulfate. Apparently, the tetrathionate formed was more easily reduced by thioglycol or sulfite, than the labily-bound iodine.

itor (mol. wt. 10,000)² is better retained. The dialyzability seems to be due to the basic nature of lysozyme, since all reactions leading to a decrease in basic groups or an increase in acid groups rendered the protein less dialyzable.

The *analytical methods* were those used previously (7, 10), in addition to the following: Tyrosine and moniodotyrosine of iodoinsulin preparations were determined spectrophotometrically by readings of the Millon-Lugg color at two wavelengths (480 and 520 $m\mu$), according to Roche *et al.* (14). Unfortunately, the colorimetric method could not be used successfully with lysozyme and its derivatives, possibly because of the high tryptophan content of this protein.

Tryptophan analyses were performed according to Horn and Jones (15) on 0.5 mg. of the unhydrolyzed sample. When different amounts of protein were analyzed, different values were obtained (7–9%) showing a definite slope which upon extrapolation to zero concentration indicated a tryptophan content of 10.3% (11.7% on the basis of the dry isoelectric protein containing 18.6% N). The lower value of 8.0% tryptophan (or 9.1% corrected) obtained with a 0.5 mg. sample agrees more closely with that obtained with enzymatic hydrolysates (16) and by other authors (17) and is regarded as more probably correct. A slightly higher value has been obtained by a recent modification of the colorimetric method (18). In contrast to findings of others (19, 20), we observed no interference by iodoproteins or their hydrolysates in the development of the color by tryptophan. When it was attempted to apply the Millon reaction according to Lugg, lysozyme yielded turbid solutions.

Cystine was determined by the Sullivan method after hydrolysis with a mixture of concentrated hydrochloric and 95% formic acids (1.0 and 0.8 ml., respectively, per 10–40 mg. protein). It appears probable that destruction of cystine was not completely prevented (17, 18). The absence of mercapto groups was concluded from negative nitroprusside tests, even in saturated guanidine hydrochloride or urea solution.

The *microbiological assays* of tyrosine and histidine were kindly performed by Dr. J. C. Lewis and Miss N. S. Snell, using *Leuconostoc mesenteroides* (P-60). Details of the methods will be published in connection with the amino acid composition of lysozyme and other egg proteins (18). The diiodo derivatives of histidine and tyrosine and also moniodotyrosine, were found inactive, but moniodohistidine was almost equivalent to histidine in the microbiological assays (88%).

Paper chromatography was used to detect the presence and purity of iodotyrosines and iodohistidines. For the former, hydrolysates prepared in sealed tubes with 8% barium hydroxide (16 hr. at 100°) were adjusted to approximately pH 4 with hydrochloric acid and extracted with butanol until the aqueous phase disappeared. The butanol extracts in turn were repeatedly extracted with a few milliliters of 0.02–0.05 *N* sodium hydroxide and the latter extract was lyophilized and taken up in a small amount of water (0.5 ml./50 mg. original protein). When a baryta solution, containing 0.5 mg. each of tyrosine, moniodotyrosine, and diiodotyrosine, was treated in this manner, all three components were evident upon chromatography of the final extract.

Hydrolysis with hydrochloric acid (5.3 *N*, 125°, 16 hr.) was used for the detection of histidine and its iodination products. The acid was evaporated off repeatedly in a

² H. Fraenkel-Conrat, Ross C. Bean, E. D. Ducay, and H. S. Olcott. In preparation for press.

desiccator. For chromatography, the solutions were applied in the usual manner to sheets of Whatman No. 1 filter paper. A mixture of butanol (200 ml.), acetic acid (30 ml.), and water (75 ml.) served as the solvent for ascending chromatography (16 hr.). After drying in air, the sheets were sprayed with 2.5% sodium carbonate and 15 min. later with an ice-cold solution of diazobenzene sulfonic acid (0.05%) in water. Spots varying from red to yellow appeared within a few seconds on an almost white background and were quite stable. Characteristic R_f values will be listed, as observed under those conditions.

Lysozyme assays were performed according to Alderton *et al.* (5, 21) by turbidimetric comparison of the rate of lysis over a 3-min. period (6 readings) of a suspension of killed *Micrococcus lysodeikticus* in $M/15$ pH 6.2 phosphate buffer when treated with various amounts of crystalline lysozyme (best range 0.005–0.02 mg./8 ml. suspension) or its derivatives. With most preparations, at least 5 of the 6 readings agreed within 10–15%, and their average, as compared with standards of similar activity, could generally be reproduced within 10%. All preparations were assayed repeatedly at several levels.

RESULTS

Acetylation, Esterification, Sulfation, or Treatment with Glucose, Formaldehyde, Periodate, or Diazobenzene Sulfonic Acid

Most of the amino groups of lysozyme are apparently essential for its activity. Thus their acetylation, succinylation, substitution with $-\text{CH}_2-\text{NH}-\text{CO}-\text{CH}_3$ (formaldehyde and acetamide) (10), or condensation with glucose (22) leads to inactivation, which appears marked even if only about one-half of the amino groups are blocked (Table I). The mode of action of the acetylated derivative differs from that of the original protein, as indicated by differing lysis-rate curves. Upon prolonged storage of a dialyzed solution of acetyl lysozyme at 3°, the protein separated as up to 2-mm.-long needles.

Esterification of the carboxyl groups with methanol or with propylene oxide leads to inactivation, the extent of which depends on the extent of reaction.⁴ Sulfation of the aliphatic hydroxyl groups by means of concentrated sulfuric acid (23) abolishes most of the activity; part of the indole groups were also destroyed by this reaction. The blocking of amide, guanidyl, and possibly a few aromatic and heterocyclic residues by means of formaldehyde in the presence of amines

⁴ Attempts to saponify the methyl ester and thereby regenerate the activity were only partly successful with 0.01 N HCl (46, and 10% activity, respectively, as compared with 40 and 6% originally), but not with 0.01 N NaOH. Trypsin used as esterase for the same purpose seemed to regenerate 0.7 acid groups from the more intensively treated preparation within 30 min. at pH 7.8 and 24°. After isolation the product retained 1.8 methoxyl groups, but only 3% of the original activity.

TABLE I
Activity of Lysozyme Derivatives

| Reaction ^a | Protein groups involved ^a | Activity % |
|--|--|---------------------------------------|
| Acetic anhydride (130) (65) | Amino (4.3) (2.6) | (8) ^b (30) ^b |
| Succinic anhydride (120) | (3.6) | 2 |
| Glucose pH 7.6, 2 days, 53° | Amino (1.35) | 1 |
| Formaldehyde (320) pH 3.7, 2 days | Amino (3.1), etc. ^c | 10 |
| Formaldehyde (320) + acetamide (850) pH 3.8, 2 days | Amino (4.9) | 22 |
| pH 5.1, 2 days | Amino (4.4) | 11 |
| pH 6.6, 2 days | Amino (3.9) | 1 |
| Formaldehyde (320) + alanine (560) pH 4.0, 2 days | Amide, guanidyl, etc. ^c (9.7) | 1 |
| pH 5.9, 2 days | (10) | 0.5 |
| Formaldehyde (320) + proline (420) pH 3.9, 2 days | Amide, guanidyl, etc. ^c (2.0) | 8 |
| Formaldehyde, pH 11.5, 10 min. | Amide, guanidyl, indole ^c | 2 ^d |
| Periodate (31), pH 5.5, 3 hr. | Indole (1.4), disulfide (1.1) | (2) ^b |
| (13), pH 5.5, 2 hr. | (0.4), disulfide | 36 |
| (31), pH 5.5, 8 M urea, 30 min. | (2.3), disulfide (0.6) | 55 |
| (31), pH 5.5, 8 M urea, 60 min. | (3.6), disulfide (1.6) | 29 |
| Performic acid, 15 min. | disulfide (2.4) | 1 |
| Diazobenzene sulfonid acid (0.7), pH 7.6, 10 min. | Phenol, imidazole | 87 |
| (1), pH 7.6, 10 min. | Phenol (0.2), imidazole (0.1) | 87 |
| (2), pH 7.6, 10 min. | Phenol imidazole | 44 |
| (3), pH 7.6, 10 min. | Phenol (0.6), imidazole (0.3) | 33 |
| (5.4 and 16) | Phenol (1.5), imidazole (0.45) | 0 |
| Sulfation (conc. H ₂ SO ₄) 10 min. -18° | Aliphatic OH (6.3) | 2 |
| Iodoacetamide, pH 11.5, 10 min. | | 69 ^d |
| Esterification (methanol-HCl) -3°, 3 days | Carboxyl (1.2) | 40 |
| 23°, 1 day | Carboxyl (3.5) | 6 |
| Propylene oxide pH 4.3, 6 days | Carboxyl | 37 |
| Ultrasonic-treated | Indole (0.5) | 100 |
| Untreated lysozyme | Amino 5.2, carboxyl approx. 6 Guanidyl 6.6, phenol 1.8 Imidazole 0.6, indole 4.5 Amide 11.0, disulfide 2.9 Aliphatic OH 11 | |

^a Amounts of reagents and extent of reaction expressed in terms of equiv./10⁴ g. protein. For composition of unreacted protein in these terms see end of table. All analyses are given on the basis of 16.5% N (uncorrected for moisture and acid).

^b The lysis-rate curve was altered; thus no exact comparison with the standard is possible.

^c See text, also Refs. (10, 24).

^d The activity of a control (pH 11.5) was 86%.

TABLE II
Iodination of *Lysozyme* and Its Reversal^a

| Reaction | | Termination reagent ^b | Reversal reagent ^b | Product ^a content ^b | | | Activity |
|---------------------------|------------------|----------------------------------|-------------------------------|---|-----------------------------------|---------------------------------|----------|
| Iodine added ^b | Time | | | Iodine bound | Tryptophan ^c unreacted | Tyrosine ^d unreacted | |
| | <i>min.</i> | | | | | | % |
| 35 | 10 | Sulfite (16) | — | 2.0 | 3.6 | 0.6 | 43 |
| 35 | 10 | Sulfite (16) | Sulfite (16) | 1.5 | — | 0.8 | 71 |
| 35 | 10 | Sulfite (16) | Sulfite (48) | 1.5 | 3.6 | 0.9 | 62 |
| 35 | 20 | Sulfite (15) | — | 2.1 | 3.7 | 0.6 | 40 |
| 35 | 20 | Sulfite (15) | Sulfite (15) | 1.5 | 3.9 | 0.75 | 69 |
| 35 | 20 | Sulfite (15) | Thioglycol (30) | 1.6 | 3.8 | — | 56 |
| 35 | 20 | Thioglycol (30) | — | 2.5 | 3.5 | — | 38 |
| 35 | 20 | Thioglycol (30) | Sulfite (15) | 1.8 | 3.8 | — | 67 |
| 35 | 20 | Thioglycol (30) | Thioglycol (30) | 1.7 | 3.8 | — | 78 |
| 35 | 24 hr. | Thioglycol (30) | — | 3.7 ^e | — | — | 1 |
| 35 | 10 (in 8 M urea) | Sulfite (16) | — | 4.0 ^e | — | — | 1 |
| 35 | 10 (in 8 M urea) | Sulfite | Sulfite (16) | 3.9 | 2.9 | 0.1 | 1 |
| 3 | 150 | Used up | — | 1.3 | 3.7 | — | 71 |
| 4(2 X 2) ^a | 45 | Used up | — | 1.6 | 3.7 | 0.9 | 28 |
| 4 | 45 | Used up | Sulfite (2) | 1.6 | 3.7 | — | 29 |
| 6(3 X 2) | 180 | Dialysis | — | 2.4 | 3.1 | 0.5 | 5 |
| 35 | 30 (pH 6.0) | Sulfite (18) | — | 1.5 | 3.8 | — | 55 |
| 35 | 30 (pH 6.0) | Sulfite | Sulfite (18) | 1.2 | 3.6 | 0.4 | 62 |
| 35 | 240 (pH 6.0) | Sulfite | — | 1.9 | 3.7 | 0.4 | 33 |
| 6(6 X 1) | 24 hr. (pH 6.0) | Used up | — | 1.5 | 3.4 | 0.8 | 19 |
| 3(3 X 1) | 24 hr. (pH 5.2) | Used up | — | 0.5 | 3.6 | 1.1 | 43 |
| Untreated | | | | 0 | 4.0 | 1.6 | 100 |

^a All reactions were performed at pH 7.6 (unless otherwise specified) and room temperature. Many experiments were performed repeatedly and the results which usually agreed within 10% were averaged. The analyses recorded are those performed on the main fraction, *i.e.*, usually the water-soluble fraction. Amounts ranging from 15–50% became insoluble during iodination and particularly during sulfite treatment. The insoluble fractions contained always more iodine and less activity than the soluble fractions, but showed the same phenomenon of reversal (*e.g.*, after treatment for 10 min. with 35 equiv. of iodine, 2.8 equiv. of iodine were bound, and 8% of the activity retained; after treatment with sulfite, the corresponding figures were 2.0 and 23%).

The iodine was added at one time, unless indicated in parentheses; in those cases, each aliquot was added upon almost complete decolorization of the reaction mixture.

^b In terms of equiv./10⁴ g. protein (about 16.5% N). Termination reagent is that used to decolorize the excess iodine, reversal reagent that added in excess of the free iodine present.

^c Approximate tryptophan content determined by analysis of 0.5 mg. of unhydrolyzed protein by the method of Horn and Jones (15), *cf.* (18).

^d Approximate tyrosine contents were obtained by doubling the results of microbiological assays of alkaline hydrolysates (5 N sodium hydroxide, 5 hr., 120°). Hydrolysis with hydrochloric acid regenerates all tyrosine from monoiodotyrosine

(10, 24) (e.g., alanine) leads to inactivation. Formaldehyde alone, in neutral or acid solution, causes cross-linking between the amino and all the other groups mentioned above; its inactivating action is thus not surprising. At pH 11.5, addition occurs to amide, guanidyl, and indole groups (16, 24).

Oxidation with periodic acid at pH 5.5 was found to attack the indole and disulfide groups, more extensively in the presence of 8 *M* urea than in its absence. The retention of some activity in preparations retaining almost no intact tryptophan suggests that these residues may not be involved in the enzymatic activity. The retention of full activity after ultrasonic destruction of a fraction of the indole groups (25) supports this conclusion. Coupling with even small amounts of diazobenzene sulfonic acid appeared to involve both imidazole and phenolic groups, as indicated by marked decreases (up to 90%) in the histidine and tyrosine content of acid hydrolysates, when assayed microbiologically. This reaction also entailed progressive inactivation (Table I).

Iodination: Nature of Reversibly-Bound Iodine

When iodine is added at pH 7.6 in great excess, part of the protein precipitates within a few seconds. The water-soluble fraction isolated after reaction periods of 10–20 min. (terminated by reduction of the excess iodine) contains about 2 equiv. of iodine/10⁴ g. and has reduced activity (about 40%). Treatment of such preparations with an excess of sulfite or thioglycol decreases the bound iodine of the soluble fraction by 0.5–0.6 equiv. and at the same time regenerates part of the activity lost during iodination (Table II).

and over half from diiodotyrosine. In line with this, only products containing 3 equiv. or more of iodine showed appreciably lowered tyrosine contents in acid hydrolysates (1.2, as compared with 1.7). *Histidine* was destroyed during alkaline hydrolysis. Acid caused loss of iodine from mono- and diiodohistidine, and monoiodohistidine is almost equivalent to histidine, microbiologically. Thus it was not surprising that the histidine content appeared unchanged in all samples except those containing 3 or more equivalents of iodine. In these the microbiologically available histidine was reduced by 40–50%.

* These analyses are on the insoluble fraction. Reaction in 8 *M* urea for 1 hr. led to almost the same iodine content (4.1 equiv.); after 24 hr. of iodination the product contained slightly less iodine (3.9 equiv.) and only 50% of the original tryptophan. The small water-soluble fraction (after 10 min. reaction) contained 3.4 equiv. of iodine and retained 14% of the activity.

If reduction is attempted after varying time periods up to 3 days, or after isolation of the primary reaction product, intermediate amounts of iodine are released (0.2–0.3 equiv.), and the activity of the product is about 55%. After 30 min. of iodination at pH 6, partial reversal of the reaction is possible.

Regeneration of activity was revealed also by immediate assays of the diluted reaction mixture, without reduction of the excess iodine or isolation of the protein by dialysis. Thus assays made 10 and 120 min. after onset of the experiment indicated activities of 23 and 9% of the original, respectively, but after a 10-min. exposure to excess sulfite, these samples, though partly not in solution, showed activities of 38 and 15%, respectively. The reversible inactivation of lysozyme by iodine was first described by Meyer *et al.* (1, 3) and later confirmed by Alderton, Fevold, and Lightbody (11). Meyer concluded from this phenomenon that lysozyme contained —SH groups. However, the protein gives no nitroprusside test, even at high concentrations and in solutions saturated with guanidine-HCl, nor does it react with tetrathionate or iodoacetamide in saturated urea or guanidine-HCl solution at pH 7. Other authors have recently arrived at the same conclusion: —SH groups do not occur in lysozyme (17).⁵

The loss of bound iodine occurring during reactivation strongly suggests that a substitution reaction, and not an oxidative one, is responsible for the reversible inactivation by iodine. The two protein groups which can be substituted with iodine are the phenolic and the imidazole groups (8, 9, 19, 20, 26–28). Model experiments with acetyl-histidine⁶ and acetyltyrosine showed that iodination occurred readily under the conditions used with lysozyme. The iodination of acetyltyrosine yielded the diiodo derivative when iodine was added in excess (5 equiv.), but an equimolar mixture of the acetyl derivatives of monoiodotyrosine and tyrosine was obtained when 1.25 equiv. of iodine was used. Tests with both mono- and diiodotyrosine showed that these compounds were not affected by treatment with sulfite under our conditions. In contrast, at neutrality histidine is known

⁵ Reported by C. M. Proctor and S. R. Dickman, University of Utah, at meeting of Federation of American Societies of Experimental Biology, Detroit, April 1949. We have confirmed the inactivation of lysozyme by xanthidrol.

⁶ The iodination products of acetylhistidine and histidine anhydride represented mixtures from which no uniform product could be isolated. From histidine both mono- and diiodo derivatives were prepared under slightly different conditions (29).

to yield at first rather unstable N-iodo derivatives, which rearrange rapidly to stable carbon substitution products (26, 27). The N-iodo derivatives can be reduced by treatment with sulfite (26). Thus the characteristics of the reversible iodine fixation of lysozyme strongly suggest that the imino-nitrogen of the imidazole group is involved.

When a series of proteins of greatly varying histidine, tyrosine, and tryptophan contents was subjected to the same reactions as performed with lysozyme, only globin and conalbumin contained some reversibly-bound iodine (0.6 and 0.5 equiv.). It appears that the N-iodination of the histidine residues in most proteins occurs more slowly than the

TABLE III

*Comparison of Amounts of Stably Bound Iodine by Various Proteins
With Their Contents of Reactive Amino Acids^a*

| Protein | Iodine bound ^b during | | Content ^b of | | |
|----------------------|----------------------------------|-------------------|-------------------------|----------|------------|
| | about 10 min. | 10 min.- 2 hr. | Histidine | Tyrosine | Tryptophan |
| Globin | 2.0 | 3.5 | 5.5 | 1.7 | 0.6 |
| LBTI ^c | 5.1 | 3.1 | 4.6 | 0.6 | 0.0 |
| Insulin | 8.2 | 2.4 | 3.1 | 6.2 | 0.0 |
| Lysozyme | 1.6 | 1.7 | 0.6 | 1.6 | 4.0 |
| Silk fibroin | 3.5 | 4.1 | 0.3 | 6.6 | 0.2 |
| Chymotrypsinogen | 2.2 | 1.2 | 0.7 | 1.4 | 3.0 |
| Tobacco mosaic virus | 3.2 | 0.4 | 0.0 | 2.0 | 1.1 |

^a All reaction performed with excess iodine at pH 7.6 (*Methods and Materials*).

^b Equiv./10⁴ g. protein.

^c Lima bean trypsin inhibitor (preparation yet to be described; see footnote 3).

migration of the iodine to the carbon atom. That the histidine binds iodine is strongly suggested by the correlation between the histidine content and the capacity for slow iodine fixation of the series of proteins treated with a great excess of iodine at pH 7.6 (Table III).⁷ Bauer and Strauss noted that globin alone of all proteins studied at that time bound more iodine than corresponded to twice its tyrosine content (26). That other proteins containing histidine may exhibit

⁷ With the exception of silk fibroin. The slow diffusion of iodine into this fibrous protein probably leads to incomplete iodination of its reactive phenolic groups in short reaction periods. To a certain extent, this is true of all proteins, as is indicated by the continued iodination of the tyrosine residues of insulin (Table V).

TABLE IV
Paper Chromatograms^a

| Compound | Hydrolysis | <i>R_f</i> values of spots | | | |
|--|----------------------------------|--------------------------------------|-------------------------|-------------------|--------------------------------|
| A. Spots applied in weakly alkaline solution | | | | | |
| Tyrosine (tyr) | None | | | 0.32 | |
| Histidine (his) | None | 0.07 | | | |
| Monoiodotyrosine (MI-tyr) | None | | (0.35) | 0.58 | |
| Diiodotyrosine (DI-tyr) | None | | | | 0.71 |
| Tyr, MI-tyr, DI-tyr | 0.5 <i>N</i> Ba(OH) ₂ | | 0.33 | 0.55 | 0.70 |
| Lysozyme, untreated | 0.5 <i>N</i> Ba(OH) ₂ | (0.17) | 0.33 | | |
| Iodo-lysoz. (1.6–1.8 equiv. of I/10 ⁴ g.) | 0.5 <i>N</i> Ba(OH) ₂ | (0.17) | 0.34 | 0.56 | 0.70 |
| Iodo-lysoz. (4.0 equiv. of I) | 0.5 <i>N</i> Ba(OH) ₂ | 0.07 | 0.35 | | 0.68 |
| Iodo-insulin (4.0 equiv. of I) | 0.5 <i>N</i> Ba(OH) ₂ | 0.07 | 0.33 | 0.54 | 0.69 |
| B. Spots applied in weakly acid solution | | | | | |
| Tyr, his | 5 <i>N</i> HCl | 0.08 | | 0.39 | |
| MI-tyr | 5 <i>N</i> HCl | | | 0.40 | |
| DI-tyr | 5 <i>N</i> HCl | | | 0.36 | 0.57 0.71 |
| Monoiodohistidine (MI-his) ^b | None | 0.17(<i>y</i>), 0.23 | | | |
| Diiodohistidine (DI-his) | None | | | 0.49(<i>y</i>) | |
| MI-his | 5 <i>N</i> HCl | 0.09 | 0.17(<i>y</i>), 0.20 | | |
| DI-his | 5 <i>N</i> HCl | 0.08 | 0.21 | (0.50, <i>y</i>) | |
| DI-tyr, DI-his | 5 <i>N</i> HCl | 0.08 | 0.13(<i>y</i>), 0.37 | | |
| Amino acids, ^c tyr, his | 5 <i>N</i> HCl | 0.08 | | 0.43 | (0.50, <i>y</i>) |
| Same, + DI-his, MI-tyr, DI-tyr | None | 0.07 | 0.32(<i>y</i>), 0.43 | 0.56 | 0.69 |
| Same, + DI-his, MI-tyr, DI-tyr | 5 <i>N</i> HCl | 0.09 | 0.16(<i>y</i>) | 0.43 | (0.53, <i>y</i>) |
| Amino-acids, MI-his | 5 <i>N</i> HCl | 0.09 | 0.18(<i>y</i>) | | 0.53(<i>y</i>) ^d |
| Lysozyme, untreated | 5 <i>N</i> HCl | 0.09 | | 0.40 | (0.48, <i>y</i>) ^d |
| Iodo-lysoz. (1.6–1.8 equiv. of I/10 ⁴ g.) | 5 <i>N</i> HCl | 0.08 | (0.13, <i>y</i>), 0.36 | 0.45 | 0.52 |
| Iodo-lysoz. (4.0 equiv. of I) | 5 <i>N</i> HCl | 0.07 | 0.12(<i>y</i>), 0.32 | 0.44 | 0.50 |
| Iodo-insulin (4.0–9.5 equiv. of I) | 5 <i>N</i> HCl | 0.07 | | 0.40 | |
| Iodo-LBTI ^e (5.1 equiv. of I) | 5 <i>N</i> HCl | 0.06 | 0.13(<i>y</i>) | 0.40 | |

^a Ascending solvent: *n*-butanol (200 ml.), glacial acetic acid (30 ml.), water (75 ml.). Spots developed by coupling with diazobenzene sulfonic acid; spots of colors other than pink were indicated by the letter *y* (yellow), although there was no clear line of demarcation. Faint spots are in parentheses. All values are averages from 2 or more chromatograms.

^b Different preparations and chromatograms yielded either one or the other spot (0.17 vs. 0.23).

^c Approximate composition of a lysozyme hydrolysate, omitting tyrosine and histidine (composition: 40 mg. asp, 16 mg. try, 16 mg. cy-S-S-, 30 mg. arg, 10 mg. lys, 40 mg. leu, 24 mg. gly, 24 mg. ser, 4 mg. met, 2 mg. pro, 8 mg. ammonium chloride, all dissolved in 8 ml. 5.3 *N* hydrochloric acid).

^d Tryptophan yielded a faint yellow spot of *R_f* about 0.5 which was overshadowed by stronger spots in iodinated materials.

^e Lima bean trypsin inhibitor (see footnote 3).

similar behavior is here shown strikingly by the lima bean trypsin inhibitor, because of its high histidine and low tyrosine content (Table III).

It was hoped that paper chromatography of hydrolysates would yield absolute proof for the iodination of the histidine residues of proteins. A few preliminary tests, however, indicated the limitations of this approach. Diiodohistidine, prepared according to Brunings (29), decomposed to both histidine and monoiodohistidine under the conditions of hydrochloric acid hydrolysis, particularly in the presence of decomposing diiodotyrosine. Monoiodohistidine appeared to occur in two modifications of slightly different R_f values, and of different color when coupled with diazobenzene sulfonic acid (both in colorimetric tests and on paper). One is yellow, similar to coupled diiodohistidine, the other, like histidine, of orange-pinkish color (Table IV). In view of the instability of iodohistidines and iodotyrosines, the interpretation of the paper chromatograms of acid-hydrolyzed iodoproteins is not simple. There is no doubt, nevertheless, that the chromatograms of iodinated lysozyme contain spots of orange and yellow color and of R_f values differing greatly from those given by the chromogenic amino acids of untreated lysozyme, and from the iodinated tyrosines. One of these spots appears to be identical with one corresponding to monoiodohistidine. This spot is also apparent in a chromatogram of iodinated lima bean inhibitor, which was shown analytically to contain iodohistidine, but it is absent from the chromatogram of an iodinsulin of similar iodine content, all of which is accounted for as iodotyrosines (Table IV). The occurrence of iodohistidine residues in lysozyme treated with an excess of iodine at pH 7.6 thus appears well established. The amounts present, and the ratio of mono- to diiodohistidine, cannot be determined with the present techniques, owing to the lability of the compounds under the conditions of hydrolysis.

Oxidative Action of Iodine

The evidence reported suggests that reversible iodination of the imidazole ring is responsible for the reversible inactivation of lysozyme. It has recently been shown, however, that the tryptophan residues of proteins are susceptible to oxidation by iodine in weakly alkaline solution (14). Colorimetric analyses were therefore performed of the

tryptophan content of various iodine-inactivated and subsequently reactivated lysozyme derivatives. It appeared that only about one-tenth of the tryptophan was destroyed during 20 min. of iodination, and that this effect was slightly, if at all, reversed by the sulfite or thioglycol treatment (Table II).⁸ Oxidative reactions involving other groups definitely appear to occur under all conditions of iodination used, but particularly below pH 7. Under such conditions only 17–25% of the iodine used up appears as bound in the isolated derivative instead of the 50% that would be bound if only substitution occurred. The marked inactivation of samples iodinated for prolonged time periods, particularly in acid solution, is attributed to oxidative changes (Table II). In 8 *M* urea the rate of iodine utilization is increased even at pH 5.2, 30–34% of it is bound, and most of the activity is retained.

Stable Substitution with Iodine

In contrast with the one imidazole group of lysozyme (17, 18), which appears to react rapidly with excess iodine at pH 7.6, the three tyrosine residues (17, 18) react only partially in the native protein. The iodine stably bound after 20 min. of reaction corresponds to the transformation of about half of the tyrosine to diiodotyrosine. In agreement with this calculation, about half of the original tyrosine was found in the alkaline hydrolysates of several iodinated lysozyme preparations. Others contained somewhat less unreacted tyrosine, and it therefore appeared possible that part of the iodine might be present as moniodotyrosine. The occurrence of appreciable amounts of moniodotyrosine in iodinated proteins has often been disputed (8, 9), but Roche and co-workers have demonstrated it clearly (19, 20). When Roche's spectrophotometric method for the differentiation of tyrosine and moniodotyrosine was applied to various lysozyme preparations, absurd results were obtained, possibly because of the high tryptophan content of this protein. In contrast, insulin iodinated under similar conditions for reference purposes could be successfully analyzed by this technique. It appeared that in insulin, although it is rich in both tyrosine and histidine, only the phenolic groups reacted readily with iodine. Moniodotyrosine was formed in appreciable amounts in ex-

⁸ The most severe iodination conditions used in this study (treatment with excess iodine at pH 7.6 for 24 hr. in 8 *M* urea) caused the destruction of half of the tryptophan.

periments where time or the amount of iodine was insufficient for diiodination of all the reactive tyrosine (Table V).⁹

It appeared that paper chromatography might serve to clarify which iodotyrosine was formed in lysozyme under our conditions of iodina-

TABLE V
Formation of Mono- and Diiodotyrosine During Iodination of Insulin

| Reaction conditions | | | Product ^a | | | | |
|---------------------------|-----|-------------|----------------------|----------|------------------|------------------------------------|----------------------------------|
| | pH | Time | Bound iodine | Tyrosine | Monoiodotyrosine | Calcd. diiodotyrosine ^b | Calcd. bound iodine ^c |
| | | <i>min.</i> | | | | | |
| Excess iodine (35 equiv.) | 7.6 | 1 | 7.2 | 2.2 | 0.8 | 3.2 | 7.2 |
| Excess iodine (35 equiv.) | 7.6 | 10 | 8.2 | 1.6 | 1.4 ^d | 3.2 | 7.8 |
| Excess iodine (35 equiv.) | 7.6 | 30 | 9.5 | 1.3 | 0.8 | 4.1 | 9.0 |
| Excess iodine (35 equiv.) | 7.6 | 120 | 10.6 | 1.1 | 0.4 | 4.7 | 9.8 |
| Excess iodine (35 equiv.) | 5.8 | 30 | 5.3 | 2.7 | 1.4 | 21. | 5.6 |
| 4 addns. of 2 equiv. | 7.6 | 30 | 4.0 | 3.1 | 2.1 | 1.0 | 4.1 |
| 9 addns. of 2 equiv. | 7.6 | 330 | 7.5 | 1.7 | 0.5 | 4.0 | 8.5 |

^a Isolated by precipitation with pH 5.0 acetate buffer and repeated cycles of washing, resolution, and reprecipitation with dilute buffer. In most experiments, half of the reaction mixture was treated with sulfite. In contrast to lysozyme, no loss of iodine was observed, nor any appreciable difference in the colorimetric analyses of the sulfite-treated and not-so-treated preparations. All analyses in terms of equiv./10⁴ g., on the basis of 14.6% N (untreated insulin, not corrected for moisture). The actual nitrogen contents of the iodinated preparations, as isolated and containing traces of the buffer salts were 13.8–12.6%; the uncorrected iodine contents range from 4.7–11.6%.

^b Difference between total tyrosine of untreated insulin (6.2 equiv./10⁴ g. protein) and that found colorimetrically as tyrosine and monoiodotyrosine.

^c Twofold of the diiodotyrosine plus the monoiodotyrosine values. To be compared with found values in column headed *Bound iodine*.

^d The presence of monoiodotyrosine and its absence, in detectable concentrations, from the more extensively iodinated samples were also shown by paper chromatograms (Table IV).

tion. Butanol-acetic acid has recently been shown to clearly resolve mono- and diiodotyrosine (30). We have found this a very useful solvent, since it also separates these two from tyrosine and gives good

⁹ Paper chromatograms also confirmed the occurrence of monoiodotyrosine in certain iodoinsulin preparations and its absence from others, depending on the method of preparation.

separations of histidine and its mono- and diiodo derivatives. Chromatography after barium hydroxide hydrolysis, developed by coupling with diazobenzene sulfonic acid, revealed very definite tyrosine spots and less strong spots corresponding to diiodo- and moniodotyrosine, respectively (Table IV). It thus appears that the stably bound iodine is present as diiodotyrosine and to a slightly lesser extent as moniodotyrosine.¹⁰

DISCUSSION

Lysozyme is a protein that is remarkably resistant to most denaturing agents and conditions. The fact that this stable protein is so readily inactivated by a variety of chemical reagents clearly indicates that the chemical change, rather than the conditions of reaction, are responsible for the observed losses of activity. It can thus be definitely concluded from the loss of activity during selective acetylation of the amino groups that the integrity of most of these groups is necessary for the enzymatic action of lysozyme. The same seems true for the carboxyl groups, and with less certainty for the aliphatic hydroxyl groups. For the amide, guanidyl, and indole groups no selective reagents were available. Thus it can only be concluded that they all may be essential for lysozyme activity. Dickman and Proctor have recently concluded from preliminary data with possibly specific reagents for amide groups that these are definitely involved,⁵ while some of the present findings suggest that the indole groups are not.

The iodination and coupling reactions indicate that substitution of the imidazole and part of the phenolic groups of lysozyme lowers the activity, and that extensive substitution abolishes it. Lysozyme in which the imidazole group is iodinated is less active than after reversal of this reaction. The phenolic groups of lysozyme are very unreactive, as compared with those of most proteins. The same is true for its

¹⁰ It appeared not worthwhile to attempt quantitative analysis of the proportion of the two compounds in view of the instability, particularly of the moniodotyrosine. Thus treatment with 5 *N* NaOH for 5 hr. causes the release of iodine from about one-fifth of the moniodotyrosine present. Diiodotyrosine is more stable in alkali. It appears noteworthy, however, that this compound (10 mg.) is up to about 60 per cent decomposed to tyrosine upon treatment with 1 ml. 5-6 *N* hydrochloric, but only up to about 8% with 1 ml. 6 *N* sulfuric acid (18 hr. at 125°). This unexpected behavior is responsible for a partly erroneous concept of the nature of the stable bound iodine of lysozyme presented at the Detroit meeting of the Federation of American Societies of Experimental Biology (April, 1949).

disulfide bonds. Both types of groups are quite reactive if the protein is dissolved in 6–10 *M* urea.¹¹

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A sample of moniodotyrosine, originally from Dr. C. R. Harington, Cambridge, England, was kindly placed at our disposal by Dr. C. H. Li of the University of California. Valuable advice concerning methods of chromatographic separation and detection of the iodotyrosines by Dr. A. Taurogg of the University of California is also gratefully acknowledged.

SUMMARY

Lysozyme is inactivated by reactions affecting its amino, carboxyl, amide, guanidyl, or hydroxyl groups. Chemical attack on the indole groups appears less harmful. Mercapto groups do not occur in lysozyme. An acetyl derivative was obtained in crystalline form.

The action of excess iodine at pH 7.6 leads to some loss of activity, which is partly regenerated in the early stages by reducing agents. The imidazole group participates in the reactions; iodination of a nitrogen atom probably accounts for the reversible inactivation. Only about half of the phenolic groups react under such conditions, and these appear to play a minor role in the activity of the enzyme.

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Day-to-Day Variation of Reduced Ascorbic Acid Content of Mare's Milk¹

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INTRODUCTION

Assays of individual samples of mare's milk have shown that when freshly drawn, the milk contains a large amount of reduced ascorbic acid. Rasmussen, Bogart, and Maynard (1938) found 27–115 mg. of reduced ascorbic acid/q. of milk; and values of 95 mg./l. have been reported by Cimmino (1938), 86–161 mg. by Holmes and Jones (1947), 91–162 mg. by Holmes (1949a) for pooled mare's milk, and 87–197 mg./l. by Cimmino (1940). These data indicate that mare's milk contains several times as much reduced ascorbic acid as freshly drawn raw cow's milk. Holmes and Jones (1947) have shown that the reduced ascorbic acid in mare's milk is far more stable than that in cow's milk; *i.e.*, when stored together in the dark at 10°C., cow's milk loses reduced ascorbic acid six to ten times as fast as does mare's milk. The observation that reduced ascorbic acid occurs in mare's milk in unusually large quantities and that it is very stable raised a question concerning the extent to which the amount of reduced ascorbic acid in the milk of individual mares varied from day to day; this study was undertaken to answer that question.

EXPERIMENTAL

Four purebred Percheron mares and one purebred Morgan mare were used in this study. The experimental period was 36 days. It was felt that this period was of sufficient duration to yield an accurate picture of the reduced ascorbic acid content of mare's milk for the stage of lactation and conditions under which it was studied.

Milk was collected from the first four mares (Fig. 1) from July 22 to August 26, 1948, inclusive. During this time, the three Percheron mares were being fitted for showing, and therefore were kept indoors in the day time and were fed three times

¹ Contribution No. 750, Massachusetts Agricultural Experiment Station.

daily early cut, mixed mature hay *ad lib.* and 4 qt. of equal parts of crushed oats and bran moistened with water and molasses. At night the mares grazed in a luxuriant pasture of rapidly growing mixed grasses. The Morgan mare received the same treatment except that her grain ration was only about one-fourth that of the others. Milk was obtained from Bay State Konhysop's Lady from February 9 to March 17, 1949. She was confined indoors continuously and was fed good quality, early cut, mixed hay *ad lib.* and 4 qt. of oats and corn daily. The collection of milk from Bay State Lena in 1949 was from August 8 to September 13, which was essentially the same period as in 1948. The ration and management conditions were the same as in 1948. Thus provision was made for comparing the reduced ascorbic acid content of milk produced under comparable conditions during successive lactation periods.

The mares were milked at about 9 a. m., and the samples were assayed within 2 or 3 hr. of that time. The amount of reduced ascorbic acid in the milk samples was determined by the procedure used by Holmes (1949b) in a study of the loss of reduced ascorbic acid from lactose-enriched milk.

RESULTS AND DISCUSSION

Approximately 150 samples of freshly drawn mare's milk were assayed for reduced ascorbic acid content. The values that were obtained are reported graphically in Fig. 1. The six curves presented in Fig. 1 have been arranged in chronological order. Thus the curve which reports the results obtained for milk produced by Narcissa (Morgan) was placed in close proximity to those for Lousop, Lady Lou, and Lena (in 1948) (Percherons) since the experimental period was the same; *i.e.*, at each of the collection days a sample was obtained from each of the four mares and the four samples were assayed simultaneously. The experimental period for Konhysop's Lady was 7 months after that of the four mares just mentioned, and the period for the repeat test with Lena (in 1949) was 1 yr. later than that of the first test. Inspection of the curves which report the results of the assays of milk produced by the three Percheron mares, Lousop, Lady Lou, and Lena (in 1948), reveals that while the amount of reduced ascorbic acid in the milk was not the same for all three mares, the day-to-day variation in reduced ascorbic acid followed the same general trend. The amount of reduced ascorbic acid in the milk from the Morgan mare and the trend followed during the study were quite different from those of the Percheron mares. As stated above, milk was collected from the four mares on identical dates, but the Morgan mare, Narcissa, was in the early stage of lactation and the Percheron mares were at mid-lactation stages. Also the Percherons were fed a larger amount of oats and bran than the Morgan mare. The reduced ascorbic acid content of the milk from Konhysop's

Lady was larger than that of the Morgan mare and less than that of the other Percheron mares; her milk, however, was produced during the winter months when she received only dry hay and concentrates, whereas the other Percheron mares were allowed grass *ad lib.* during nights in an excellent pasture. Also the day-to-day variation in the amount of reduced ascorbic acid in this mare's milk did not follow the pattern of that of either the Morgan or the Percheron mares previously discussed.

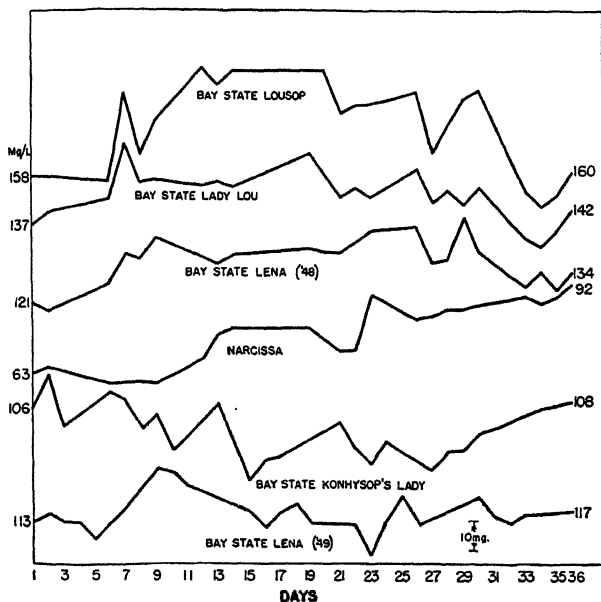


FIG. 1. Variation of reduced ascorbic acid in mare's milk.

The day-by-day variation in the reduced ascorbic acid content of milk produced by Bay State Lena in 1949 differed from that produced by the same mare under comparable conditions the previous year. Also, at each of the 25 assay periods, the milk produced in 1949 contained less reduced ascorbic acid than the milk produced at the corresponding period in 1948. These observations raise a question concerning the extent of repeatability of the results obtained with animals under standardized experimental conditions. The question of whether duplicate results can be obtained in repeat experiments has received attention by

investigators who have studied different species of the animal kingdom. Sidwell and Grandstaff (1949), in a study of the size of lambs at weaning as a permanent characteristic of Navajo ewes, concluded that the repeatability coefficient for weaning weights of lambs raised by Navajo ewes was 0.217; Lush and Molln (1942), who were interested in the litter size and weight as permanent characteristics of sows, obtained a value of 0.18 for repeatability of productivity, which was similar to values reported by other investigators cited by these authors.

The data reported here indicate very definitely that the results obtained from a single sample of a mare's milk may not supply very accurate information. Inspection of the graphs reveals that there was considerable day-to-day variation in the amount of reduced ascorbic acid found in the milk of each of the five mares used. It might be assumed that since Lousop, Lady Lou, and Lena (in 1948) were maintained under standardized, uniform conditions, the milk from any of the three would contain approximately the same amount of reduced ascorbic acid from day to day, but such was not the case. For instance, if the reduced ascorbic acid content of the milk from these three mares had been judged by the amount found in the initial samples, erroneous conclusions might have been deduced. In fact, since the reduced ascorbic acid content of the initial and final samples, collected 36 days apart, are in good agreement for each of the three mares, it would be assumed erroneously that the amount of reduced ascorbic acid in mare's milk was uniform for the individual animal. Actually, even though the initial and final samples of milk produced by Lousop contained 158 and 160 mg. of reduced ascorbic acid/l., respectively, the average amount of reduced ascorbic acid for all the samples of milk collected from Lousop during the 36-day period was 175 mg./l. Likewise, the milk produced by Lady Lou and Lena (in 1948) exhibited the same trend. The results reported in Fig. 1 show that the amount of reduced ascorbic acid in mare's milk varies almost daily. Consequently, if information is desired regarding the average reduced ascorbic acid content of mare's milk over any considerable period, it is necessary to collect and assay samples of the milk at fairly frequent intervals during the period under discussion.

SUMMARY

Samples of milk were collected from a Morgan mare and four closely related Percheron mares in order to secure data regarding the extent of day-to-day variation in the amount of reduced ascorbic acid in mare's

milk. The experimental period was 36 days, and 23-25 samples of milk were collected from each mare. One mare was used during the same stage of two lactations. Identical results were not obtained for these two lactations, and the extent to which duplicate results can be obtained in repeat experiments of this type was discussed. The data assembled from each of the six lactations show that the amount of reduced ascorbic acid in mare's milk fluctuates significantly and the value obtained from a single sample of mare's milk may lead to quite erroneous conclusions. To secure an accurate picture of the average amount of reduced ascorbic acid in mare's milk, it is essential to collect and assay samples of milk at relatively frequent intervals during the period under consideration.

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The Isolation of Adenosine Triphosphate from Plant Tissue¹

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INTRODUCTION

The key role which high-energy phosphorus compounds play in mechanisms of energy transfer and biosynthesis made it seem of primary importance to demonstrate their occurrence in plants and to devise methods for their isolation and estimation as the prerequisite to plant-growth studies.

In an earlier publication (1), one of us reported the presence of labile phosphorus in acid-soluble extracts of oat seedlings, suggestive of the possible presence of compounds like adenosine triphosphate (ATP). Oats had been chosen as a preliminary material because of the extensive studies, on mechanisms of growth under the influence of auxins, which had been made with this material. By a modification of Needham's (2) procedure, we succeeded in isolating from germinated oat seedlings an adenine-pentose-pyrophosphate in low yield and of low purity (3). This nucleotide appeared to be related to the adenosine diphosphate (ADP) of animals but was not identical with it.

The high polysaccharide content and the presence of large quantities of phytic acid and of nonspecific ultraviolet absorbing material in oat extracts which made it difficult to follow the compound through successive isolation steps stimulated the search for another plant material which could be more easily handled.

Mung beans were finally chosen² because:

¹ This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and by a grant from the American Philosophical Society.

² We wish to thank Professor S. F. Trelease of Columbia University for suggesting the use of mung beans.

1. Mung bean sprouts are available in large quantity as an article of commerce.³

2. They are relatively low in storage carbohydrate and phytic acid.

3. Crude extracts of the material exhibit a clear cut ultraviolet spectrum with a maximum at 2600 Å and relatively little end absorption.

This report outlines a procedure for the isolation of an adenosine triphosphate of approximately 70% purity from mung bean sprouts.

METHODS

Inorganic, labile, and total phosphorus were determined according to the procedure of Fiske and SubbaRow (4) as modified in Umbreit, Burris, and Stauffer (5). Pentose was determined according to the method of Mejbaum (6) with a 45-min. heating time as recommended by Albaum and Umbreit (7). Adenine was measured spectrophotometrically in the Beckman spectrophotometer. Adenylic acid and adenosine triphosphate, used as purine standards for the spectrophotometric and enzymatic runs, were obtained from the Ernst Bischoff Company, Ivoryton, Connecticut.

Rates of deamination of adenosine triphosphate, both plant and animal, were measured in the spectrophotometer at 265 mμ according to the procedure of Albaum and Lipshitz (8). The procedure, briefly, consists of converting adenosine triphosphate into adenylic acid with a combined hexokinase-myokinase system and measuring the adenylic acid formed with a muscle deaminase. Muscle deaminase (Schmidt's deaminase) was prepared according to the method of Kalckar (9); myokinase was prepared according to Colowick and Kalckar (10). Hexokinase was prepared in partially pure form according to Berger, Slein, Colowick, and Cori (11), through step 3 (fractionation with ethanol at 0°C.) of the isolation procedure.

In the studies of the half reactions (hexokinase and myokinase) described above, the same enzymes were used; extent of transfer of phosphorus from ATP to glucose was measured by calculating the disappearance of labile phosphorus from the different substrates (plant and animal ATP) with time. The enzyme reactions were stopped by the addition of 1 ml. of 5 *N* H₂SO₄ containing 2.5% ammonium molybdate.

The chromatographic runs were carried out in 6-in. by 18-in. pyrex jars on Schleicher and Schüll No. 597 paper cut to 15 × 17 in. size, rolled into cylinders, and stapled on top and bottom. The migration was upward in a developer consisting of 1% malonate buffer at pH 6, mixed with approximately $\frac{1}{3}$ of a volume of isoamyl alcohol; migration was allowed to take place for a period of 16 hr., at room temperature. After drying, the nucleotides were detected and *R_f* values determined by direct observation with a mineralight No. 44 (Ultraviolet Products Inc., Los Angeles, Calif.). (We are indebted to Dr. George B. Brown of the Sloan Kettering Institute for suggesting the procedure.)

EXPERIMENTAL

In preliminary experiments on the isolation of adenosine triphosphate from mung bean sprouts, various procedures which had been used suc-

³ These are grown in large quantity for the Chinese restaurant industry.

cessfully in this laboratory for the isolation of ATP from animal sources were tried. These included the methods of Kerr (12), of Needham (2), and Szent-Györgyi (13). We also tried the procedure which Kiessling and Meyerhof (14) had used for the isolation of a di-adenine nucleotide from yeast. All these yielded unsatisfactory results when applied to the plant tissue and gave preparations which appeared to be partially split and extremely low in yield.

The following procedure has consistently yielded preparations of approximately 70% purity. For convenience the procedure is subdivided into four steps.

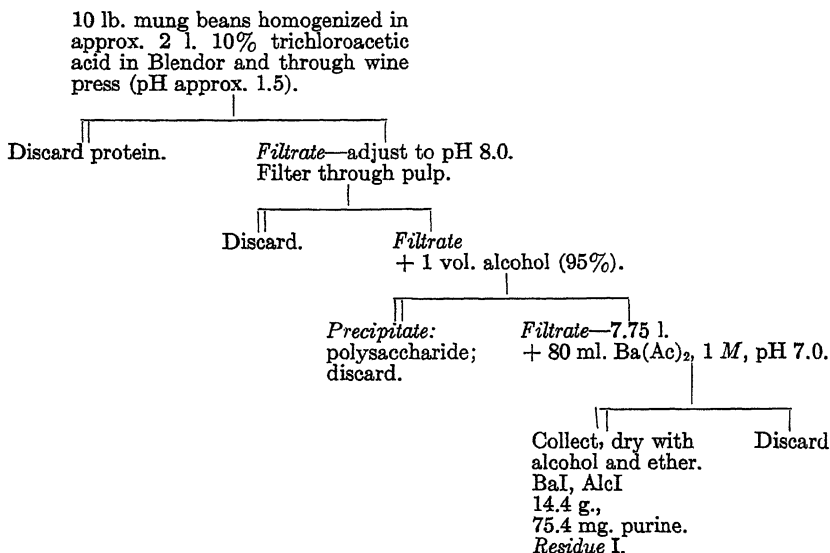


FIG. 1. Preparation of residue I.

Step 1, the preparation of a barium-insoluble, alcohol-insoluble residue (I) is outlined in Fig. 1 for a 10-lb. batch of beans. These had been germinated for 5 days in the dark and had hypocotyls approximately 2-in. in length.

Parallel studies of samples of residues I prepared from mung beans and from rabbit muscle help to point up some of the difficulties encountered in applying existing isolation procedures devised for animal muscle

to plant tissue. Ninety-seven per cent of the adenine⁴ of the rabbit muscle residue was extracted at pH 2.6 as compared with approximately 25% from the plant material. Even when the plant residue was suspended in water and the pH adjusted to 1, it was found that most of the adenine remained with the insoluble residue after centrifugation. Since this residue is normally discarded in existing procedure (*e.g.*, that of Needham for animal tissue), a part of the background leading to the low yields in plant tissues was illuminated.

Since more than 90% of the inorganic phosphate of residue I was also extractable at pH 2 under conditions where the bulk of the adenine

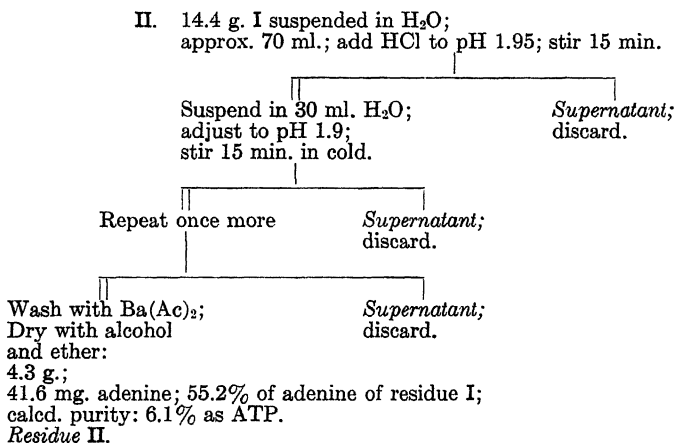


FIG. 2. Preparation of residue II.

material was still insoluble, a fractionation at pH 2, leading to the elimination of the bulk of the inorganic P, was carried out, resulting in residue II (Fig. 2).

Residue II still contained 60% of the adenine originally present in residue I. The purity of ATP at this stage calculated from the adenine was approximately 7%. The ratio of adenine: labile P: total P in this crude preparation was found to be 1.0: 1.85: 3.24. This preparation was deaminated in the hexokinase-myokinase-deaminase system, but at a slower rate than animal ATP. Controls in which plant and animal

⁴ Ultraviolet-absorbing material with a peak at 2600 Å has been assumed to be adenine in the isolation procedure. All calculations involving this material have been made utilizing the extinction coefficient for adenosine triphosphate.

preparations were deaminated together indicated that nothing in the plant preparation inhibited the enzymes used in the assay. The plant preparation at this stage also gave a slower rate of color development in the orcinol-pentose reaction (7) than did animal ATP.

The very low pH required to bring residue II into solution with subsequent splitting of the ATP made it impractical to dissolve the nucleotide by this method. In further purification another technique, there-

TABLE I
Fractionation after Sodium-Sulfate Exchange and Silver Treatment
Analytical data of residues III A, B, and C

| | A BaI, pH 2.0 | | B BaI, pH 7.0 (between 2-7) | | C BaS (pptd. 1 vol. alc.) | |
|--|---------------------------------|-----------|-----------------------------------|-----------|---------------------------------|-----------|
| | Average 4 runs | Range | Average 4 runs | Range | Average 4 runs | Range |
| Total wt. of ppt., mg. | 242 | 145-292 | 179 | 104-250 | 51 | 28-72 |
| Total adenine, mg. | 10.15 | 7.0-13.0 | 11.65 | 5.0-17.6 | 1.59 | 1.0-2.4 |
| Ratio: adenine | 1.00 | — | 1.00 | — | 1.00 | — |
| $\Delta 7'$ P | 2.06 | 1.91-2.18 | 1.80 | 1.76-1.88 | 0.91 | 0.90-0.91 |
| total P | 3.17 | 3.06-3.30 | 2.89 | 2.68-3.18 | 2.03 | 1.93-2.10 |
| pentose | 1.03 | — | 1.00 | — | 1.04 | — |
| Purity as ATP, per cent | 27.1 | 23.5-30.8 | 39.6 | 29.8-54.1 | | |
| Rate of deamination as compared with ATP, per cent | 66.0 | 60.7-69.8 | 63.8 | 58.6-72.8 | 46.1 | 42.8-49.7 |
| Recovery of adenine in fractions A, B, C | 64.6% of adenine of residue II. | | | | | |

fore, had to be devised to bring the barium salt into solution without lowering the pH too drastically. By stirring residue II in the cold with an excess of sodium sulfate at pH 2.0,⁵ the greater part of the adenine could be extracted as a soluble sodium salt; barium sulfate produced as a result of the exchange was centrifuged off. To obtain complete exchange it was found necessary to use an excess of sodium sulfate, some

⁵ This is not the usual method of removing barium. The "usual" method involves adding stoichiometric quantities of Na_2SO_4 . In the presence of stoichiometric quantities of Na_2SO_4 the exchange in these insoluble residues is only partially effective.

of which still remained after the removal of the barium sulfate. The presence of this sulfate made it impractical to precipitate the nucleotide as the mercury salt. Silver, however, could be used. The addition of silver as the nitrate, followed by decomposition with hydrochloric acid

- III. 4.22 g. II suspended
in 78 ml. 0.2 *N* HNO₃
+ 10 ml. *M* Na₂SO₄;
stirred 15 min. in cold.

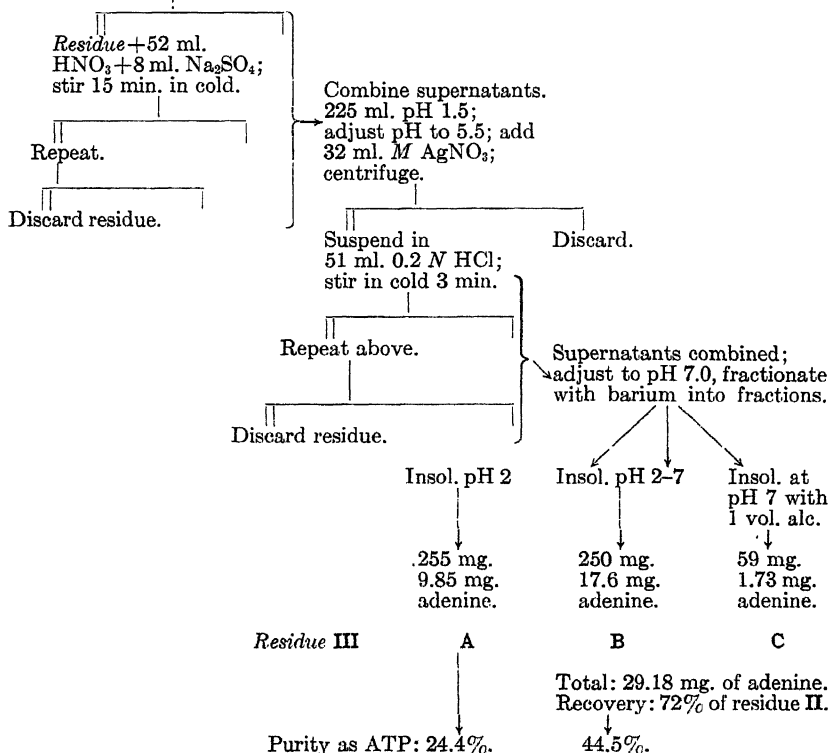


FIG. 3. Preparation of residue III.

proved to be satisfactory. Precipitation with barium at this point yielded from a fraction initially insoluble at pH 2.0, three fractions, one still precipitable as the barium salt at pH 2.0, a second insoluble between pH 2 and 7, and a third precipitable with one volume of alcohol at pH 7.0.

Table I shows the analytical data for these fractions. Fractions **IIIA** and **IIIB** analyze as ATP, whereas **IIIC** appears to be principally ADP. Both the purity and the deamination rates of fractions **IIIA** and **IIIB** have increased markedly over that of residue **II**. Further purification has been carried out thus far only on fraction **IIIB**, since it was soluble and could be handled readily. Studies on the nature of fraction **IIIA** have been deferred to a later time. The preparation at this stage was yellow in color and showed some absorption at 320 m μ . The procedure used in further purification is shown in Fig. 4 (residue **IV**).

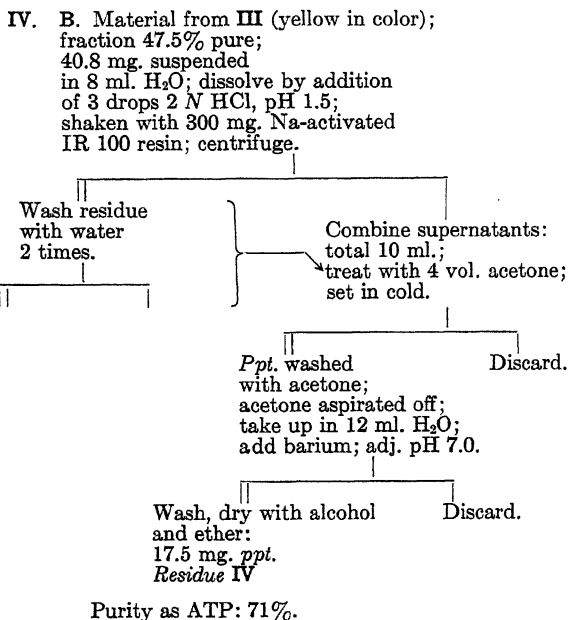


FIG. 4. Preparation of residue **IV**.

Table II shows the analytical data on a sample of residue **IV** in another run. As the result of the step just outlined, residue **IV** was now white in color, showed no absorption at 320m μ , assayed 64% pure as ATP with the characteristic molar ratio of adenine, labile P, total P, and pentose. The purification had brought its rate of color development in the orcinol-pentose reaction (7) to a level comparable with animal ATP but had not appreciably affected the slow rate of deamination in the complete enzyme system.

In order to account for this behavior of the plant ATP in the enzyme tests, the preparation was subjected to a number of other procedures.

First, deamination runs again were carried out on plant and animal preparations, alone and in combination. These were set up so that the aliquots were identical with respect to adenine, labile, and total P. The results are shown in Table III. It is apparent that since the change in the combined aliquots is the same as the total of the individual aliquots, the slow rate of observed deamination in the plant material cannot be attributed to inhibitors which would tend to inhibit the enzyme systems used. By the same token, since all the adenine and phosphorus in the plant preparation are accountable for as ATP,

TABLE II

Analytical Data for Residue IV

Combined B fractions (BaI, pH 7.0) from 3 different batches; 500 mg. purified with acetone and amberlite; final yield: 118 mg. barium salt.

| | |
|---|-----------|
| Total salt, <i>mg.</i> | 118 |
| Adenine, <i>mg.</i> | 11.1 |
| Purity as ATP, <i>per cent</i> | 64.0 |
| Ratio: adenine | 1.00 |
| 7' P | 2.16 |
| total P | 3.08 |
| pentose | 0.98 |
| Rate of deamination, <i>per cent</i> | 30.6/47.0 |
| | 65.2% |
| Color of animal ATP | |
| in 7' pentose reaction, <i>per cent</i> | 62.4/62.3 |
| | 100% |

one cannot ascribe the results obtained to the presence of adenine or phosphorus contaminants.

Second, to test whether or not deamination merely proceeded more slowly in the plant preparation, the reactions were carried to completion with both the plant and animal ATP of equivalent adenine content, as shown in Fig. 5. Whether examined from the point of view of adenylic-acid deamination or from inosinic-acid production, the plant preparation is deaminated to the extent of only approximately 60% of the animal ATP.

Third, in view of the slow deamination reported by Kiessling and Meyerhof (14) on a di-adenine nucleotide isolated from yeast with an

alkali labile linkage postulated between the stable P of ATP and the 3-C of a 5-adenylic acid, the plant preparation (residue IV, Fig. 4) was treated with 0.0025*N* NaOH for 5 min. at 100°C. No resultant increase in deamination was observed.

Fourth, to test for any evidence of a di-nucleotide link involving C-2 or C-3 in the plant material, the periodate oxidation of Lythgoe and Todd (15) was applied. No difference was observed between plant and animal ATP in the periodate titration.

In view of the fact that the deamination system employed was based upon removal of the labile P of ATP by means of hexokinase and myokinase, the partial reactions with the latter enzymes were examined to see whether they were responsible for the differences observed be-

TABLE III
*Course of Deamination of Plant and Animal Adenosine Triphosphate
with a Combined Hexokinase-Myokinase-Deaminase System*

| | Decrease in density at 265 mμ | | | |
|---|-------------------------------|------|------|------|
| Time, min. | 4 | 8 | 12 | 16 |
| Plant ATP (Residue IV) | .007 | .016 | .027 | .037 |
| Animal ATP | .014 | .029 | .045 | .063 |
| Total | .021 | .045 | .072 | .100 |
| Plant and animal ATP run together in same tube | .021 | .049 | .074 | .100 |

The aliquots used in this experiment contained identical amounts of adenine, labile P, and total P.

tween plant and animal ATP in rate and extent of deamination. Table III records the results of a typical run in terms of the disappearance of labile P in 20 min. It will be noted that the plant ATP transfers approximately half as much labile P in each of two systems as does the animal ATP; this suggests the possibility that half of the labile P in the plant ATP is blocked in some fashion.

As a further test for differences in plant and animal ATP, their migration on a paper chromatogram was studied, using 1.0 *M* malonate at pH 6.0 saturated with isoamyl alcohol. Mean data of 30 replicate runs are shown in Table V. The difference in *R_f* values between plant and animal ATP, though small, appears to be highly significant, as evidenced by a deviation/standard error of more than 7.

Further purification of the plant material was attempted by precipitating the mercury salt, decomposing with hydrogen sulfide and reprecipitating as the barium salt. At earlier stages in the isolation, purification through a mercury step invariably resulted in splitting, with no signifi-

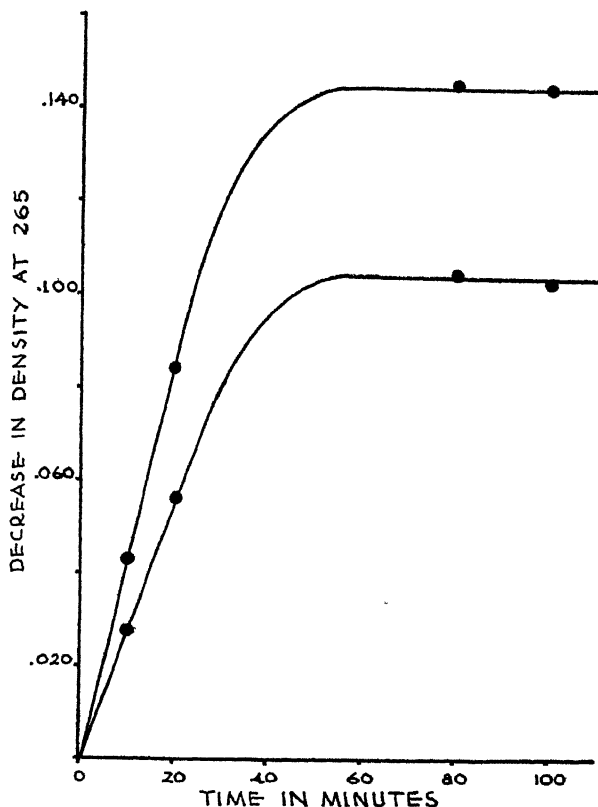


Fig. 5. Time curve of deamination of adenylic acid formed from adenosine triphosphate in presence of complete hexokinase-myokinase system. Upper curve: animal adenosine triphosphate; lower curve: plant adenosine triphosphate, fraction IV. See text for experimental details.

cant yield of ATP. The results of carrying residue IV through mercury purification are shown in Table VI. Only about one-third of the ATP was recovered although animal ATP can be carried through this step with only small losses. The resultant material exhibited only slightly

TABLE IV

Per Cent Labile P Transferred in 20 Min. to Glucose^a

| | | |
|------------|---------------------------|----|
| Animal ATP | hexokinase | 45 |
| | hexokinase plus myokinase | 87 |
| Plant ATP | hexokinase | 23 |
| | hexokinase plus myokinase | 47 |

^a See text for details.

TABLE V

Chromatographic Data R_f Values^a

| | <i>mean</i> | <i>n</i> | <i>σ</i> |
|------------|-------------|----------|----------|
| Plant ATP | .846 | 32 | .019 |
| Animal ATP | .873 | 37 | .014 |

d/S.E.^b 7.2^a Residue IV was used in these experiments. See text for details.^b Ratio of deviation to standard error.

TABLE VI

Attempt at Further Purification of Residue IV

Pure material (64%) fractionated once with $\text{Hg}(\text{Ac})_2$ in acetic acid; decomposed with H_2S ; barium salt formed.

| | |
|---|-----------|
| Total salt: fractionated, <i>mg.</i> | 30.0 |
| recovered, <i>mg.</i> | 10.0 |
| Recovered: total adenine, <i>mg.</i> | 1.08 |
| initial adenine, <i>mg.</i> | 2.84 |
| Purity as ATP, <i>per cent</i> | 68.2 |
| Ratio: adenine | 1.02 |
| 7' P | 2.00 |
| total P | 3.03 |
| pentose | 1.03 |
| N | 5.08 |
| Rate of deamination | 64.9/69.0 |
| | 94.5% |
| Color in 7' pentose reaction as | 63.7/62.3 |
| compared to animal ATP, <i>per cent</i> | 102.0% |

enhanced purity but its deamination rate was now more nearly comparable with animal ATP. At this point, on the basis of the criteria we have been using, it was identical with animal ATP.

DISCUSSION

It is possible to isolate from mung bean sprouts an ATP of approximately 70% purity which is indistinguishable from the ATP isolated

from rabbit muscle and other animal sources. The fact remains, however, that conventional procedures for the isolation of ATP from animal sources which have been in regular use in this laboratory are unsuccessful when applied to the plant tissue. The differences in behavior of the plant ATP at several stages in the isolation procedure which has been devised seem more suggestive of an additional structural link blocking reactive groups rather than of the presence of an inhibiting impurity in the preparation.

With the plant experience in mind we have re-examined the first barium precipitate from rabbit muscle in the Needham procedure after repeated extraction in cold 0.2 *N* nitric acid and found that some ATP was still bound in the residue and could be brought into solution with acidified sodium sulfate. In a study of the distribution of radioactive phosphorus in various phosphorus-containing compounds of the cyclophorase gel, one of us (16) has applied this technique to demonstrate another adenine polyphosphate fraction, not identical with that normally containing ADP and ATP, which was highly radioactive.

The literature contains a number of references to adenine pentose polyphosphates with linkages other than those known in the linear triphosphate proved by Lohmann (17) and synthesized by Baddiley, Michelson, and Todd (18). Structural possibilities involving an additional link to ribose such as suggested by Kiessling and Meyerhof (14) and Umbreit and Le Page (19) seem to be excluded by the similar results obtained in the periodate oxidation of the plant and animal ATP's. Recently, Michelson and Todd (20), in an attempt at the synthesis of an isomer of ATP by an unambiguous synthesis, obtained the linear triphosphate rather than the expected isomer. They have suggested a trimetaphosphate as the intermediate between the two forms and have speculated on the extent to which such a compound might be expected to occur naturally.

The general question is raised as to the extent to which all or part of the ATP in various tissues of both plants and animals is free, or linked to structural components, or internally bound.

SUMMARY

1. A procedure is described for the isolation of adenosine triphosphate from mung bean sprouts with a purity of approximately 70%.

2. At this stage of purity, it differs from animal adenosine triphosphate in the rate at which deamination occurs in a hexokinase-myokinase-deaminase system. This slow rate of deamination is associated with the unavailability of one-half of the labile phosphorus for transphosphorylation.

3. A compound identical with animal adenosine triphosphate may be isolated from purified fractions with mercury treatment.

4. The possible structural significance of these findings is discussed in terms of anomalous properties of adenosine triphosphate encountered in plant and animal tissues.

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Studies of the Fate of Radioactive 2,4-Dichlorophenoxyacetic Acid in Bean Plants¹

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INTRODUCTION

As part of a study of plant growth regulators which is under way here, 2,4-dichlorophenoxyacetic acid (2,4-D) labeled in the carboxyl group with C¹⁴ has been synthesized and applied to young red-kidney bean plants. The movement of radioactive carbon has been studied by determining its distribution in the plant at various times after application of the radioactive 2,4-D, and the metabolic fate of 2,4-D in these plants is being investigated. It has been found that nearly all of the radioactive material present in the plants 1 week after treatment can be isolated from the plants. The first stages in the determination of the chemical identity of this material are reported in this paper.

MATERIALS AND METHODS

Synthesis of Radioactive 2,4-Dichlorophenoxyacetic Acid

Carboxyl-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) was synthesized in 74% yield from 0.4 mmole of carboxyl-labeled sodium acetate.² The sodium acetate (one mcurie of C¹⁴/mmole) was placed in an evacuated system, dry hydrogen chloride was admitted and maintained at atmospheric pressure, and the solid was heated. The acetic acid, purified by repeated low pressure sublimation in Dry-Ice traps, was condensed in a reaction tube made from one part of a $\frac{3}{8}$ 10/30 joint. Red phosphorus (0.5 mg.) and dry bromine (0.04 ml.) were added and the mixture was heated (condenser jacket placed on the tube) for 1 hr. on a micro hot plate maintained at 100°C. The mixture was protected from the atmosphere by a trap maintained below 0°C. The mixture was then heated to reflux temperature, the hot plate was removed,

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² Purchased from Tracerlab, Inc., Boston, Mass., on allocation from the U. S. Atomic Energy Commission.

and the remaining bromine was distilled into the cold trap by reducing the pressure. To the crude bromoacetic acid was added a solution of 400 mg. of 2,4-dichlorophenol and 112 mg. of sodium hydroxide in 0.5 ml. of water, and the resulting solution was heated at 100°C. for 6 hr. The solution, diluted with water, was saturated with carbon dioxide, and was extracted repeatedly with ether. The aqueous solution was acidified and was extracted repeatedly with ether. The ether solution was washed with 1 ml. of water, and the ether was distilled at atmospheric pressure; residue: 77 mg. (dried *in vacuo*). The radioactive 2,4-dichlorophenoxyacetic acid was recrystallized from 0.8 ml. of benzene followed by the addition of 4 ml. of petroleum ether; weight: 70 mg.

Non-radioactive 2,4-dichlorophenoxyacetic acid, prepared identically, melted at 138–9°C. (micro, corrected) [reported m. p. 138° (1)] and had the following analysis: C, 43.64%; H, 2.72%; Cl, 32.44%; neutralization equivalent, 223. Analysis calculated for $C_8H_6O_3Cl_2$: C, 43.47%; H, 2.74%; Cl, 32.08%; neutralization equivalent, 221.

Treatment of Bean Plants

The red-kidney bean plants (*Phaseolus vulgaris* L.) were grown under artificial, controlled conditions. Seeds were germinated in Petri dishes in the dark. After 60 hr. the seedlings were placed in Hoagland's nutrient solution and were grown under fluorescent light of 750–1000 foot-candles intensity with 17 hr. of light/day. The temperature was maintained at approximately 27°C. and the relative humidity at 30–35%.

Preliminary experiments with young red-kidney bean plants had indicated that 10 μ g. of 2,4-D applied, in 0.5% Carbowax 1500 solution, to one primary leaf on the midrib near the petiole caused pronounced physiological responses but did not kill the plants. Therefore, for application to the plants in the experiments described here, 0.50 mg. of the radioactive 2,4-D was dissolved in 1 ml. of 0.5% Carbowax 1500 solution and 0.020 ml. of this solution was applied to each plant. The plants were treated when the primary leaves were almost fully expanded but the terminal bud was still very small (approximately 9 days after germination). The plants were treated approximately 5 hours after the beginning of the 17-hr. light period.

Radioactivity Measurements

The samples were oxidized using Van Slyke-Folch combustion solution (2). In the case of plant fractions the complete fractions were oxidized in order to eliminate sampling errors. Using the procedures described by Keller, Rachele, and du Vigneaud (3, 4), the carbon dioxide was absorbed in sodium hydroxide solution, barium carbonate was precipitated by the addition of barium chloride solution, and the barium carbonate was collected on 21-mm. discs of Schleicher and Schuell No. 589 blue-ribbon filter paper. A lead-shielded Tracerlab thin mica window Geiger counting tube was used with a Nuclear Instrument Model 161 scaling unit to measure the radioactivity of the barium carbonate discs.

In most instances the barium carbonate samples were of "infinite thickness" (5), and, therefore, the observed radioactivity minus background was directly proportional to the concentration of radioactive carbon. In the few instances in which the

amount of material was insufficient to give a sample of barium carbonate of "infinite thickness" the observed radioactivity was corrected to the value for "infinite thickness" using an empirical self-absorption curve.

RESULTS AND DISCUSSION

Distribution of Radioactive Carbon in the Plants after Treatment with 2,4-D

Twelve plants were treated (10.0 ± 0.5 μ g. of radioactive 2,4-D/plant), and 4 plants were harvested after 6 hr., four after 2 days, and the remaining four after 7 days. Each plant was divided into fractions,

TABLE I
Radioactivity of Various Plant Parts
(counts/min.)

| Plant part | Plants harvested after 6 hr. | | | | Plants harvested after 2 days ^a | Plants harvested after 7 days ^a |
|---|------------------------------|-------------|-------------|-------------|--|--|
| | Plant no. 1 | Plant no. 2 | Plant no. 3 | Plant no. 4 | | |
| Terminal bud | 4.3 | 3.0 | 0.6 | 2.8 | 12.8 ± 3.1 | 3.8 ± 1.0 |
| Untreated primary leaf | 0.7 | 1.2 | -0.1 | 0.8 | 0.3 ± 0.05 | -0.1 ± 0.5 |
| Petiole of untreated primary leaf | 1.3 | 0.3 | 0.0 | 0.9 | 0.8 ± 0.5 | 0.4 ± 0.4 |
| Treated primary leaf minus treated area | 0.2 | -0.5 | -0.1 | 1.3 | 0.9 ± 0.5^b | 1.3 ± 0.8 |
| Petiole of treated primary leaf | 23.8 | 14.9 | 11.2 | 17.7 | 16.2 ± 3.3 | 12.5 ± 3.3 |
| First internode | 22.0 | 20.1 | 8.8 | 13.0 | 28.8 ± 4.0 | 12.7 ± 3.8 |
| Hypocotyl | 7.0 | 5.3 | 2.6 | 4.3 | 21.3 ± 4.6 | 11.7 ± 4.4 |
| Roots | 0.6 | 0.3 | 1.9 | 1.0 | 3.7 ± 0.4 | 2.9 ± 0.8 |
| Root tips (combined) | 0.2 | | | | 1.6 | 1.0 |

^a Mean of 4 plants \pm mean deviation.

^b Mean of only 2 plants.

the fractions were dried at room temperature in a vacuum desiccator over phosphorus pentoxide at approximately 20 mm. pressure, and weighed. (A negligible amount of radioactive carbon was present in the drying agent at the end of this procedure.) The carbon present in each fraction was converted to barium carbonate for measurement of the radioactive carbon present. The results are given in Table I. The values recorded are observed counts/min. minus background. The probable error of these values due to counting is ± 0.4 counts/min.

The data in Table I indicate the relative concentrations of radioactive carbon in the various plant parts; an observed value of 22 counts/min. corresponds to the presence of the radioactive carbon from 0.1 μ g. of 2,4-D/3 mg. of total carbon in the plant part. The highest concentrations of radioactive carbon occur in the petiole of the treated primary leaf, in the first internode, and in the hypocotyl. The data in the table do not indicate the total amount of radioactive carbon in each plant part since the total amount depends on the size of the plant part as well as on the concentration of radioactive carbon.

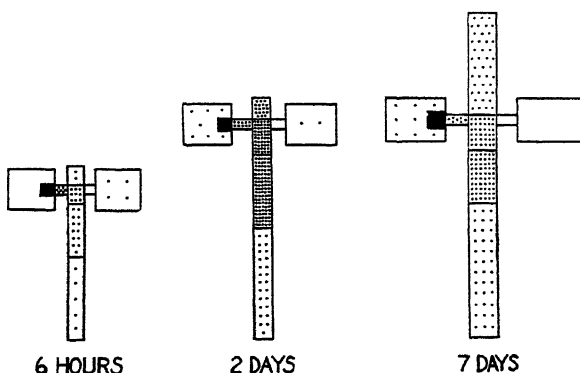


Fig. 1. Schematic representation of the distribution of C^{14} in young red-kidney bean plants harvested 6 hr., 2 days, and 7 days after treatment with radioactive 2,4-D. The sections of the central column of each diagram represent, from top to bottom, the terminal bud, first internode, hypocotyl, and roots. The area of each section is drawn proportional to the average dry weight of the plant part. The black area on the left primary leaf of each plant is the place where the 2,4-D was applied. Each black dot represents the amount of radioactive carbon present in 0.01 μ g. of 2,4-D.

In order to show all of these data, the averages, at each time period, of the concentration of radioactive carbon, the dry weight of the plant part, and the total amount of radioactive carbon in each plant part are given in Fig. 1.

It is clear from the data in Fig. 1 and Table I that the movement of radioactive carbon in red-kidney bean plants, under the conditions used in these experiments, is from the treated primary leaf through the petiole and then chiefly downward in the stem, eventually reaching the roots. A smaller amount of radioactive carbon moves up into the

terminal bud and practically none moves into the other primary leaf. Aside from this general pattern of movement of the radioactivity, considerable variations were observed among the individual plants. For example, the amount of 2,4-D absorbed varied from 25 to 60% even though the application of the 2,4-D was the same and the major environmental factors were closely controlled.

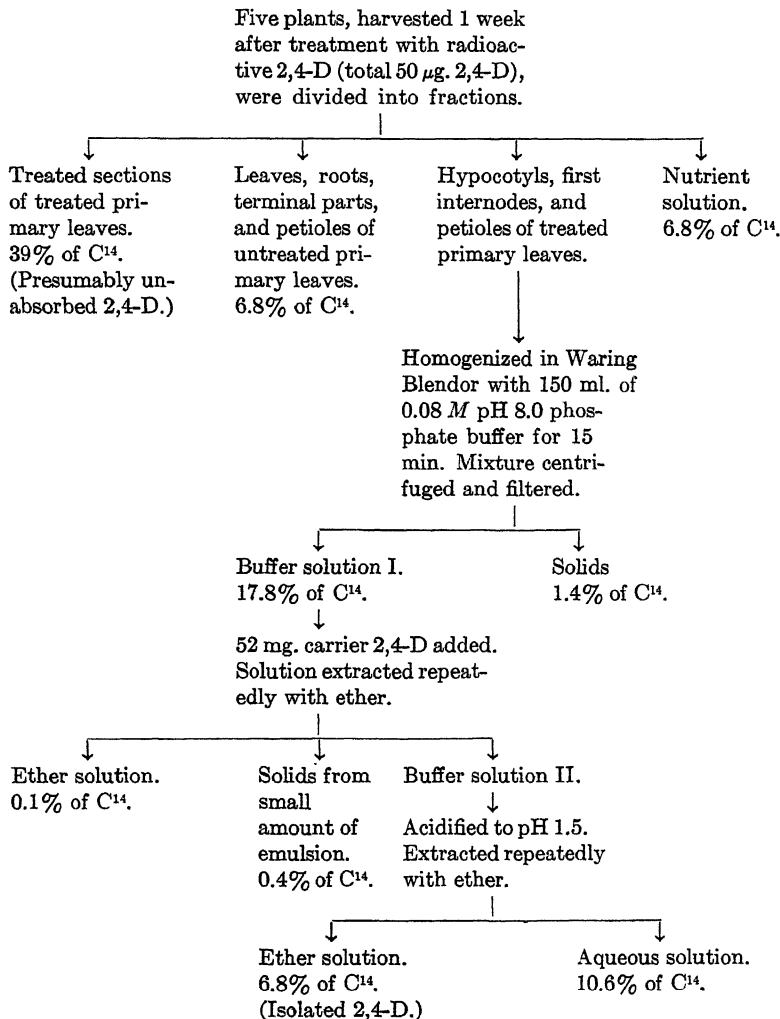
The movement of radioactivity after the application of labeled 2,4-dichlorophenoxyacetic acid is different from the movement of radioactivity, studied by Mitchell and co-workers (6, 7), after the application of labeled 2-iodo-3-nitrobenzoic acid. These workers found that the radioactivity concentrated in the terminal bud of bean plants. Recently, Mitchell and Linder (8) have found that the radioactivity from 2,4-dichloro-5-iodophenoxyacetic acid, labeled with radioactive iodine, accumulates mainly in the upper hypocotyl and first internode of bean plants. This is in close agreement with the data reported here. It indicates that 2,4-dichlorophenoxyacetic acid and 2,4-dichloro-5-iodophenoxyacetic acid behave similarly and that both have behavior different from that of 2-iodo-3-nitrobenzoic acid.

Isolation of Radioactive Material from Plants Seven Days After Treatment

Five plants were harvested 7 days after treatment with radioactive 2,4-D. The plants were subjected to the procedure outlined below. Only the stems were used in the isolation of radioactive material as they contained most of the radioactive carbon, and when the complete plants were used there were difficulties with emulsions. The amount of radioactive carbon which was found in each fraction is given in the outline.

It will be noted in the outline that a considerable amount of the radioactive carbon was not accounted for at the end of 1 week. This had been observed previously in the studies of the movement of radioactive carbon in the plants. It has been established that radioactive carbon dioxide is given off by treated plants, at least in the dark, though this has not yet been placed on a quantitative basis for the 1-week growing period.

The isolated 2,4-D (carrier 2,4-D plus any radioactive 2,4-D present in the buffer solution) was purified by subjecting it to a 24-plate

Procedure for Isolation of Radioactive Material from Bean Plants

counter-current distribution in the Craig apparatus (9, 10, 11) using ether and 1.0 M pH 5.8 phosphate buffer as the two phases. The results of the counter-current distribution are shown in Fig. 2. The weight curve is chiefly 2,4-D (added carrier) with small amounts of

impurity at each end. The central portion of the curve representing radioactivity follows the 2,4-D curve closely. (A control experiment indicated that the radioactive 2,4-D was free of any impurity which could be separated by the counter-current distribution technique.) The central portions of both curves fit calculated theoretical curves (12) within experimental error. The presence of small amounts of at least two ether-extractable radioactive organic acids other than 2,4-D is established by the presence of radioactive carbon in the end tubes of the counter-current distribution.

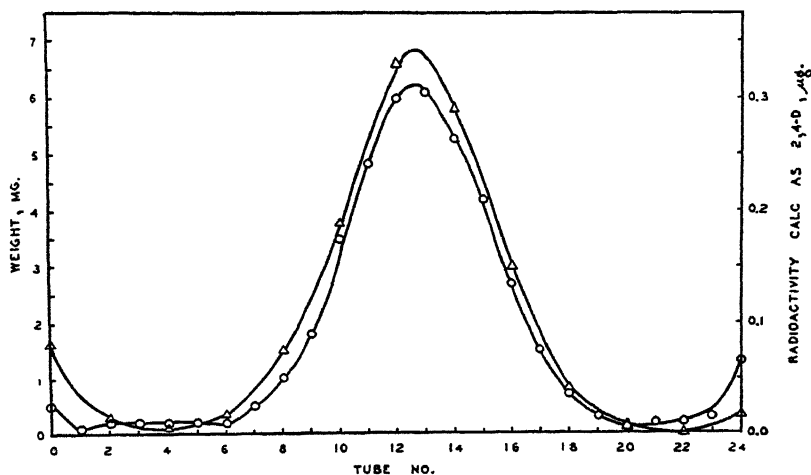


FIG. 2. Results of 24-plate counter-current distribution of isolated 2,4-D: o, weight; Δ , radioactivity.

After the radioactivity measurements on the fractions of the counter-current distribution were complete, the 2,4-dichlorophenoxyacetic acid remaining in tubes 10–16 was combined (24.3 mg.) and recrystallized three times from 10 ml. of ligroin (90–110°). Part of the purified 2,4-D [total recovery 22.8 mg., micro m. p. 138–9° (corr.)] was used in a radioactivity measurement. The remainder (16.4 mg.) was mixed with 0.5 ml. of thionyl chloride and the solution refluxed for 5 min. The excess thionyl chloride was removed at reduced pressure. To the residue was added 0.05 ml. of aniline and, after about 1 min., a few milliliters of absolute ether. The ether solution was washed with 1 *N*

hydrochloric acid and with water, and the ether solution (free of water droplets) was distilled to dryness. The weight of unpurified 2,4-dichlorophenoxyacetanilide was 20.8 mg. (95%). This material was recrystallized twice from 3 ml. of ligroin (90–110°) to give 12.0 mg. of pure 2,4-dichlorophenoxyacetanilide, micro m. p. 107.5–110° (corr.). Part of this material was used in a radioactivity measurement.

The results of radioactivity measurements at various stages (Table II) indicated that the specific radioactivity of the isolated 2,4-D was constant within experimental error. It may be concluded that 2.7 μ g. of radioactive 2,4-D (5.4% of the applied 2,4-D) was present in buffer solution I. Therefore, approximately one-third of the total amount of radioactive material in the buffer solution was 2,4-D. Of greater interest is the demonstration that two-thirds of the radioactive material in the buffer extract was not 2,4-D. The major portion of this material is water-soluble and cannot be extracted by ether from an aqueous solution at pH 8.0 or at pH 1.5. An investigation of this material is in progress.

TABLE II

Amount of Radioactive 2,4-D in Buffer Solution I

| | |
|---|--------------------------|
| Calculated after distribution of 2,4-D | 2.90 \pm 0.10 μ g. |
| Calculated after three recrystallizations of 2,4-D | 2.70 \pm 0.10 μ g. |
| Calculated after two recrystallizations of the anilide of 2,4-D | 2.73 \pm 0.10 μ g. |

SUMMARY

1. Carboxyl-labeled radioactive 2,4-dichlorophenoxyacetic acid (2,4-D) has been synthesized and applied to red-kidney bean plants.

2. The distribution of radioactive carbon in the plants at various times after application of 2,4-D to the leaves indicates that the chief movement of radioactive carbon is downward in the stems. Radioactive carbon is lost from the plants as carbon dioxide and is also lost to the nutrient solution.

3. A procedure has been devised which makes possible the isolation of nearly all the radioactive material present in the stems of treated plants.

4. Approximately one-third of the isolated radioactive material is 2,4-D.

5. Over half of the isolated radioactive material is not 2,4-D, is water-soluble, and cannot be extracted by ether from an aqueous solution at pH 8.0 or pH 1.5.

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Fluorescence of Coumarin Derivatives as a Function of pH

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INTRODUCTION

The fluorescent properties of organic compounds have, until recently, been badly neglected as a tool in chemical and biological research. Fluorescence may be used as a means of detecting, identifying, and quantitatively determining traces of such compounds. Since the fluorescence of substances in aqueous solutions is usually greatly influenced by hydrogen ion concentration, data on changes in the color and intensity of fluorescence of a compound over a wide range of pH should be useful to those interested in fluorometric methods. In fact, pH-fluorescence curves may be as characteristic as absorption spectra and have the advantage that they may be obtained on relatively impure preparations or extracts (11).

The present paper reports data on the fluorescent behavior of 98 different coumarin derivatives. The authors first became interested in these compounds after they had been able to isolate and identify one of the blue-fluorescing substances present in oat (*Avena sativa* L.) roots as scopoletin (7-hydroxy-6-methoxycoumarin) (9). Part of the evidence for the probable identity of the *Avena* substance with scopoletin was the identity of the pH-fluorescence curves. The certainty of the identification would be improved if it could be shown that scopoletin had a pH-fluorescence curve distinct from that of other coumarin derivatives. For this reason it seemed desirable to determine the color of the fluorescence and the shape of the pH-fluorescence curve for as many coumarins related to scopoletin as could be obtained. None of the coumarins which we have thus far tested had a pH-fluorescence curve that would be confused with that of scopoletin. It was hoped further

that the shape of some one of these pH-fluorescence curves might give a clue as to the identity of other unknown compounds present in *Avena* extracts.

A few other ways in which the data on the fluorescence of the coumarins may be useful are indicated at the end of this paper.

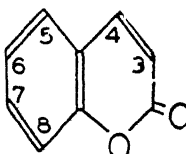
METHODS

The methods employed were essentially the same as those we have previously described (11). The substances investigated were used as received without further purification, unless otherwise noted. They were dissolved in water or a small volume of hot ethanol and diluted to a suitable concentration with water. Then 1.00 ml. of stock solution was put into a cylindrical cuvette and diluted to the 5-ml. mark with buffer. The final concentration was chosen so that the maximum fluorescence gave a scale reading of about 100 on the Klett fluorometer¹ when the instrument was set to read 100 above the buffer blank with a 1 mg./l. quinine sulfate standard in a cylindrical cuvette. The following Corning filter combinations were used: lamp filter 5860 + 738 and photocell filter 3060; or lamp filter 5860 and photocell filter 3060 + 3389. The same values were obtained for esculin and scopoletin solutions with either filter combination.

The buffers used were: 8 *N* sulfuric acid (pH - 1.6), 4 *N* sulfuric acid (pH - 0.7), 1.5 *N* sulfuric acid (pH 0.3), 0.1 *N* sulfuric acid (pH 1.3), MacIlvaine citrate-phosphate buffers (pH 2.2-8.0), mixtures of 0.2 *M* Na₂HPO₄ and 0.2 *M* Na₂CO₃ (pH 9-10), 0.2 *M* Na₂CO₃ (pH 11.0), 0.010 *N* NaOH (pH 11.7), 0.10 *N* NaOH (pH 12.6). The pH values were those obtained after diluting with the solution tested, as described above. All pH values were measured with glass electrodes with the exception of the three most acid values, which were obtained from the data of Michaelis and Granick (13). All measurements of fluorescence at pH 11 and higher were made immediately after diluting with buffer. Whenever there was evidence of decomposition or synthesis of the fluorescent material, the measurements were repeated.

RESULTS

Information obtained concerning the nature of the fluorescence of the coumarin derivatives tested is summarized in Table I and Figs. 1 to 3.

Coumarin has the structure , where the numbers represent

the positions on the molecule where substitutions may be made.

¹ The senior author is indebted to the Rumford Committee of the American Academy of Arts and Sciences for a grant to cover the purchase of this instrument.

The structure and position of the substitutions or the structural formula of each compound is given in the table. References to the biological distribution of the naturally-occurring compounds are included at the end of the table. In column 2, numbered footnotes refer to sources of the samples, and lettered footnotes refer to the fluorescent behavior of the compounds. In column 3, the fluorescence is expressed as a scale reading which would have been obtained for a concentration of the compound of 1 μ mole/l. at the pH giving maximum fluorescence. At the right, pH-fluorescence curves are given for all compounds having an appreciable fluorescence. To obtain these curves the relative fluorescences were computed by dividing the fluorescence (potentiometer scale reading) at each pH by the maximum fluorescence and multiplying by 100.

The color of the fluorescent light was blue throughout the pH range unless noted otherwise in the footnotes, but there may have been unnoticed changes in the shade of blue light with changes in pH. Any increase or decrease of fluorescence during irradiation in the fluorometer has also been recorded as a footnote.


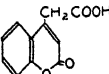
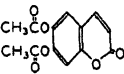
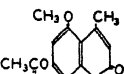
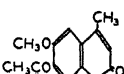
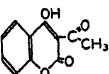
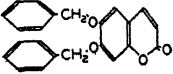
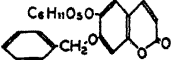
In Table I, a maximum fluorescence of <1 could be caused by the presence of $<1\%$ of a strongly fluorescent impurity. In deciding whether or not some of the low fluorescences (e.g., coumarin-3-carboxylic acid and 7-hydroxy-8-acetyl-4-methylcoumarin) were caused by impurities, the shape of the pH-fluorescence curves of the substance and related compounds were considered. Several of the compounds about which there was doubt were recrystallized and the measurements repeated.

Data on the Fluorescence of Coumarin Derivatives

In naming derivatives, priority has been given to substitutions in the 7-position. Derivatives are arranged in alphabetical order with the following exceptions: compounds with methyl substitutions appear immediately after their unmethylated analogs; compounds with ring systems other than that of coumarin are listed at the end of the table. The following abbreviations are used: COOH, carboxy; COOEt, carbethoxy; diMe, dimethyl; diMeO, dimethoxy; diOH, dihydroxy; Et, ethyl; EtO, ethoxy; Me, methyl; MeO, methoxy; OH, hydroxy. Special names of derivatives which have been isolated from plants appear often after the name of the substitution, and the species of plants from which they have been obtained are given in a separate paragraph following the table.

Numbered footnotes refer to the sources of our samples. Lettered footnotes refer to the fluorescent behavior of the compound. The color of the fluorescence was blue unless otherwise stated in the footnotes.

TABLE I

| NAME OF COMPOUND OR SUBSTITUTION | | FOOTNOTES | MAXIMUM FLUORESCENCE |
|----------------------------------|---|------------|-------------------------|
| Coumarin |  | 1, a | <0.006 |
| METHYL COUMARINS | | 2, 3, b | <0.002 |
| 4-ACETIC ACID |  | 4 | 0.005 |
| 6,7-DIACETOXY |  | 5, c | 294 * |
| 6,7-DIACETOXY-4-Me | | 5, c | 231 * |
| 7-ACETOXY-5-MeO-4-Me |  | 6, c, d, e | 274 * |
| 7-ACETOXY-6-MeO-4-Me |  | 5, e | 300 * |
| 3-ACETYL-4-OH |  | 4 | 0.015 |
| 7-BENZYLOXY-6-ACETOXY-4-Me | | 5, f | 186 * |
| 6,7-DIBENZYLOXY |  | 5 | 57 * |
| 6,7-DIBENZYLOXY-4-Me | | 5 | 44 * |
| 7-BENZYLOXY-6-GLUCOXY |  | 5, g | 21 * |
| 7-BENZYLOXY-6-GLUCOXY-4-Me | | 5 | 13 * |

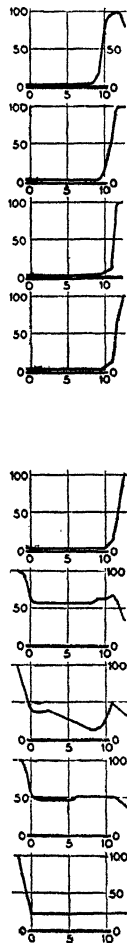


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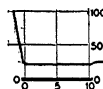
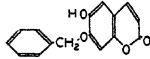
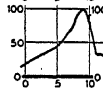
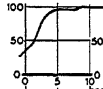
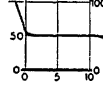
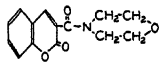
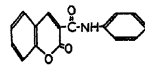

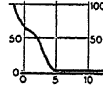
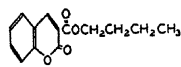
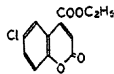
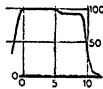
| NAME OF COMPOUND OR SUBSTITUTION | FOOTNOTES | MAXIMUM FLUORESCENCE |
|---|-----------|--|
| 7-BENZYLOXY-6-TETRAACETYLGLUCOXY | 5, g | 21*  |
| 7-BENZYLOXY-6-OH  | 5, h | 0.603  |
| 7-BENZYLOXY-6-OH-4-Me | 5, i | 1.62*  |
| 7-BENZYLOXY-6-MeO-4-Me | 5 | 46*  |
| 3-(4-CARBONYL MORPHOLINE)  | 7 | <0.005 |
| 3-CARBOXANILIDE  | 7, j | 0.067 |
| 3-COOH  | 8, k | 0.82  |
| 3-COOH-8-MeO | 9, m | 0.16 |
| 3-n-BUTYL CARBOXYLATE  | 10, n | 0.17 |
| 4-COOEt-6-CHLORO  | 4, k | 0.54*  |
| 6-CHLORO | 4, a | 0.005 |
| 6-CHLORO-4-Me | 4 | 0.009 |
| 7-CHLORO-4-Me | 4 | 0.024 |

TABLE I (Cont.)

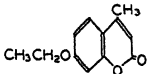
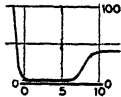
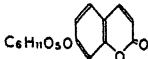

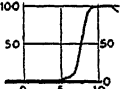
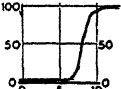
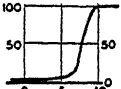
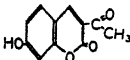
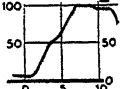
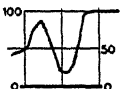
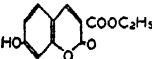
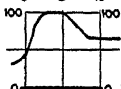

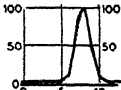

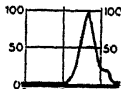
| NAME OF COMPOUND OR SUBSTITUTION | | FOOTNOTES | MAXIMUM FLUORESCENCE |
|----------------------------------|---|----------------------|---|
| 7-EtO-4-Me |  | 4 | 6.32*  |
| 7-GLUCOXY Skimmin |  | 11, n | 0.13 |
| 3-OH | | 12 | 0.032 |
| 4-OH | | 4 | 0.002 |
| 7-OH Umbelliferone |  | 13, o, p 14, o, d | 380 280  |
| 7-OH-4-Me | | 15, o | 634  |
| 7-OH-5-Me | | 16 | 331  |
| 7-OH-3-ACETYL |  | 16 | 88  |
| 7-OH-8-ACETYL-4-Me | | 17, a | 0.60 |
| 7-OH-3-COOH | | 9 | 376  |
| 7-OH-3-COOEt |  | 9, p 16, p | 302 334  |
| 5,7-diOH |  | 19, n | 11  |
| 5,7-diOH-4-Me |  | 6, p 20, p | 36* 17  |

TABLE I (Cont.)


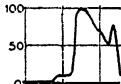
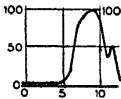


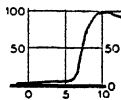
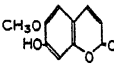
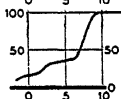
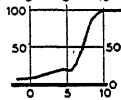
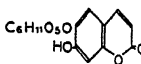
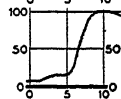
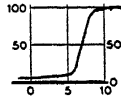

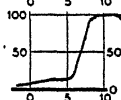
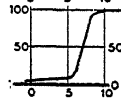
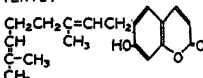
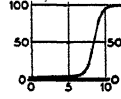
| NAME OF COMPOUND OR SUBSTITUTION | | | FOOTNOTES | MAXIMUM FLUORESCENCE | |
|---|------------|---|----------------------------------|-------------------------|---|
| 6,7-diOH | Esculetin |  | 21 | 16 |  |
| 6,7-diOH-4-Me | | | 5, o | 52* |  |
| 7,8-diOH | Daphnetin |  | 22 | 0.17 | |
| 7,8-diOH-4-Me | | | 7, k | 0.029 | |
| 7-OH-5-MeO-4-Me | |  | 6, r | 278* |  |
| 7-OH-6-MeO | Scopoletin |  | 23, o, p 23, o, p 24, o, p | 301* 301 278 |  |
| 7-OH-6-MeO-4-Me | | | 5 | 314* |  |
| 7-OH-6-GALACTOXY | |  | 5 | 333* |  |
| 7-OH-6-GALACTOXY-4-Me | | | 5 | 350* |  |
| 7-OH-6-GLUCOXY | Esculin |  | 5, p 25, p 25, p | 357* 370* 370 |  |
| 7-OH-6-GLUCOXY-4-Me | | | 15 | 345* |  |
| 7-OH-6-(3,7-diMe-2,6-OCTADIENYL) Ostruthin | |  | 11, c | 198 |  |

TABLE I (Cont.)

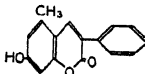
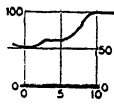
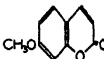
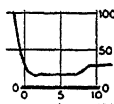
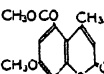
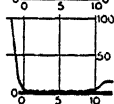
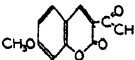
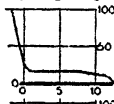
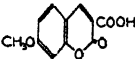
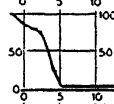
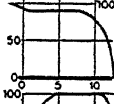
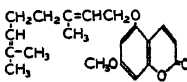
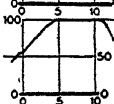
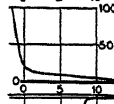
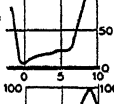
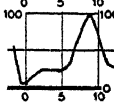
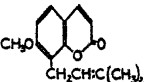

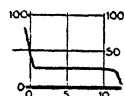

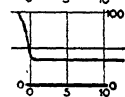
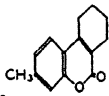
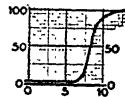


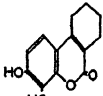

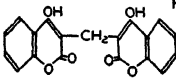
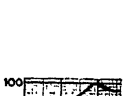
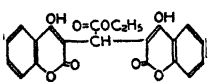
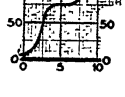
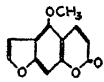

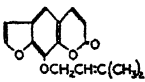
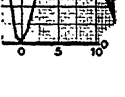
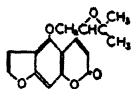
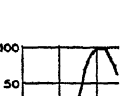
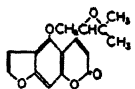
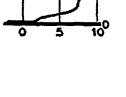
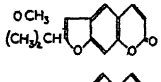

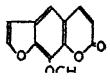
| NAME OF COMPOUND OR SUBSTITUTION | | FOOTNOTES | MAXIMUM FLUORESCENCE |
|-----------------------------------|---|-----------|--|
| 7-OH-3-PHENYL-5-Me |  | 16, s | 398  |
| 4-MeO | | 4 | <0.001 |
| 7-MeO Herniarin |  | 26, n, t | 1.26  |
| 7-MeO-5-ACETOXY-4-Me |  | 6, m | 3.2*  |
| 7-MeO-3-ACETYL |  | 16 | 0.82  |
| 7-MeO-3-COOH |  | 16 | 166  |
| 7-MeO-3-COOEt | | 16 | 118  |
| 7-MeO-5-GERANOXY |  | 2, u | 1.65  |
| 7-MeO-3-OH | | 12 | 2.44  |
| 7-MeO-5-OH-4-Me | | 6, v, w | 0.47*  |
| 7-MeO-6-OH-4-Me | | 5, x | 3.56*  |
| 7-MeO-8-(2-ISOPENTENYL) Usthol |  | 11 | 0.061 |

TABLE I (Cont.)

| NAME OF COMPOUND OR SUBSTITUTION | FOOTNOTES | MAXIMUM FLUORESCENCE |
|--|------------|----------------------|
| 5,7-diMeO Limettin | 2 | 0.046 |
| 5,7-diMeO-4-Me | 6, c, d, t | 11* |
| 5,7-diMeO-3-CH ₂ COOEt-4-Me | 16, r, v | 4.51 |
| 5,7-diMeO-3-COOEt-6-OH | 16, y | 2.25 |
| 5,7-diMeO-3-PHENYL | 16, c, v | 275 |
| 6,7-diMeO | 9, c | 56 |
| 6,7-diMeO-3-COOH | 9, c, d | 286 |
| 6,7-diMeO-3-COOEt | 9 | 278 |
| 7-MeO-3-PHENYL-5-Me | 16 | 202 |
| 8-MeO | 18, k, n | <0.013 |
| 7-n-PROPYLOXY | 9 | 5.56 |
| 7-n-PROPYLOXY-3-COOH | 9 | 98 |
| 1-THIOCOUMARIN | 27 | <0.004 |
| 2-THIOCOUMARIN | 4 | <0.002 |

TABLE I (Cont.)

| NAME OF COMPOUND OR SUBSTITUTION | FOOTNOTES | MAXIMUM FLUORESCENCE |
|--|---|---|
| 5,6-BENZOCOUMARIN |  4, c, d | 184*  |
| 7,8-BENZOCOUMARIN |  4, d, g | 28*  |
| DIBENZO[b,d]PYRONE, 3-Me-7,8,9,10- 3-Me-7,8,9,10-TETRAHYDRO |  14 | <0.007  |
| DIBENZO[b,d]PYRONE, 3-OH-7,8,9,10-TETRAHYDRO |  14, o, z | 322  |
| DIBENZO[b,d]PYRONE, 3,4-diOH-7,8,9,10-TETRAHYDRO |  14, k | 0.13  |
| 3,3'-METHYLENE-BIS-4-OH Dicoumarol |  4 28 | <0.003 <0.001  |
| 3,3'-COOEt-METHYLENE-BIS-4-OH |  4 | <0.001  |
| Bergapten |  11, c, d | 1.11  |
| Imperatorin |  11, k | 0.041  |
| Marmelosin |  6, aa | 1.8*  |
| Oxypeucedanin |  11, k | 0.043  |
| Peucedanin |  11 | 0.464  |
| Xanthotoxin |  29, bb | 0.035 |

In column 3 the fluorescence is expressed as a potentiometer scale reading for a concentration of 1 μ mole/l. at the pH giving maximum fluorescence. The actual concentration tested varied, depending upon the fluorescence of the particular compound. The highest concentration used was 500 μ moles/ml. The fluorometer scale was set to 100 above the buffer blank for a quinine standard of 1 mg./l. of 0.1 *N* sulfuric acid. The following Corning filter combinations were used: lamp filter 5860 + 738 and photocell filter 3060; or lamp filter 5860 and photocell filter 3060 + 3389. Values obtained with the second set of filters are marked with an asterisk.

The graphs at the right represent pH-fluorescence curves, with pH as abscissa and relative fluorescence as ordinate.

Sources of Compounds

1. Eastman Kodak Co., Rochester, N. Y.
2. Fritzsche Bros., N. Y.
3. Verona Chemical Co., Newark, N. J., provided a sample of 3-methylcoumarin.
4. Dr. H. Veldstra, N. V. Amsterdamsche Chininefabriek, Amsterdam, Holland.
5. Dr. G. Amiard, Services Scientifiques Roussel-Uclaf, Paris, France.
6. Dr. Hans Schmid, University of Zürich.
7. Parke, Davis and Co., Detroit, Mich.
8. Recrystallized from a sample from Dow Chemical Co., Midland, Mich.
9. Dr. Richard Baltzly, Wellcome Research Laboratories, Tuckahoe, N. Y.
10. Dow Chemical Co., Midland, Mich.
11. Prepared by Dr. E. Späth, University of Vienna.
12. Dr. A. Butenandt, University of Tübingen.
13. A Schuchardt preparation obtained through the Wellcome Research Institution, London, England.
14. Dr. Virgil Boekelheide, University of Rochester.
15. Recrystallized from a sample from Koppers Co., Pittsburgh, Pa.
16. Dr. T. R. Seshadri, Delhi University, Delhi, India.
17. Recrystallized from a sample from Parke, Davis and Co., Detroit, Mich.
18. Obtained by decarboxylation from the 8-methoxy-3-carboxylic acid derivative provided by Dr. Richard Baltzly, Wellcome Research Laboratories, Tuckahoe, N. Y.
19. Dr. J. H. Looker, Ohio State University.
20. Recrystallized from a sample from Edwal Laboratories, Inc., Chicago, Ill.
21. Prepared by hydrolysis of esculin.
22. Merck & Co., Rahway, N. J.
23. Dr. Léo Marion, National Research Council of Canada.
24. Extracted from *Artemisia* sp. and obtained from Wellcome Research Institution, London, England.
25. Eimer & Amend, N. Y.
26. Extracted from *Matricaria Chamomilla* L. and obtained from Wellcome Research Institution, London, England.
27. Dr. Floyd DeEds, U. S. D. A.
28. E. R. Squibb and Sons, N. Y.
29. Isolated and partially purified by Mr. H. Abu-Shady, University of Minnesota.

Notes on Fluorescence

* Lamp filter 5860 and photocell filter 3060 + 3389 were used.

^a Rapid conversion to a green-fluorescing compound upon irradiation in the fluorometer at pH values above 10.

^b All the monomethylcoumarins have been tested and all are nonfluorescent. The 5-methyl-, 6-methyl-, 7-methyl-, and 8-methyl- derivatives are converted into greenish-blue-fluorescing compounds upon irradiation in the fluorometer at pH 11.

^c Photolabile between pH 11.7 and 12.6.

^d Color of fluorescence greenish between pH -1.6 and 0.2; blue at higher pH values.

^e Fluorescence increases upon irradiation at pH 10.

^f Fluorescence increases rapidly upon irradiation at pH 11 and 11.7.

^g Conversion to a green-fluorescing compound upon irradiation in the fluorometer at pH 11.7 and 12.6.

^h Color of fluorescence whitish above pH 8.

ⁱ Color of fluorescence yellowish above pH 5, becoming more yellow with increasing pH.

^j Three coumarin-3-carboxanilide derivatives from Parke, Davis and Co., with sulfamyl, carboxyl, and 2-thiazolylsulfamyl groups substituted in the *para* position on the anilide ring, have also been tested and found nonfluorescent.

^k Color of fluorescence greenish.

^m Color of fluorescence greenish above pH 11.

ⁿ Fluorescence increases rapidly upon irradiation in the fluorometer at pH 11.7 and 12.6.

^o Somewhat photolabile at pH values above 9.

^p Both samples gave identical pH-fluorescence curves.

^q The shape of the pH-fluorescence curve of this preparation was nearly identical with that of 7-hydroxy-4-methylcoumarin, and could be due to the presence of 0.1% of the latter compound as an impurity.

^r Color of fluorescence greenish-blue between pH -1.6 and 0.2; deep blue above pH 1. Somewhat photolabile at pH 12.6.

^s Color of fluorescence greenish-blue at pH values above 6.

^t Photolabile at pH -1.6.

^u Very photolabile above pH 0.3.

^v Color of fluorescence greenish-yellow at pH -1.6.

^w Color of fluorescence greenish-yellow above pH 7.0.

^z Color of fluorescence whitish-blue from pH 1.2 to 6.2; green at pH 11.7 and 12.6.

^{aa} Color of fluorescence lavender at pH -1.6 and -0.7; pink from pH 0.3 to 7.0; lavender at pH 8.0; blue from pH 9.0 to 12.6. The pink fluorescence when examined with a spectroscope was observed to contain a considerable amount of red light of wavelengths longer than 620 mμ.

^{ab} Compare this 3,4-substituted 7-hydroxycoumarin with umbelliferone.

^{ac} Color of fluorescence greenish-yellow.

^{bb} Color of fluorescence greenish in acid and bluish in alkaline solutions.

Occurrence of Compounds in Nature

Bergapten is a constituent of bergamot oil and occurs in the fruit of *Fagara xanthoxyloides* Lam. (29).

Coumarin occurs in seeds of *Diptherix odorata* Willd. and *Myroxylon Pereirae* Klotzsch.; in blossoms of *Melilotus officinalis* (L.) Lam.; in shoots of *M. alba* Desr., *M. infesta* Guss., *Liatris odoratissima* Michx., *Asperula odorata* L., *Anthoxanthum odoratum* L., *Angraecum fragrans* Thou., *Orchis fusca* Jacq., *Achlys triphylla* DC., and *Lavandula officinalis* Chaix. (3).

Daphnetin occurs as the glucoside daphnin in the bark of *Daphne Mezereum* L. and in *D. Gnidium* L., *D. alpina* L. and *D. Laureola* L. (5).

Dicoumarol has been isolated from spoiled sweet clover (*Melilotus alba* Desr.) hay (6).

Esculetin occurs in the bark of *Aesculus Hippocastanum* L. (16), *A. turbinata* Blume (17), and in the seeds of *Euphorbia Lathyris* L. (27).

Esculin occurs in the bark of *Aesculus Hippocastanum* L., in the roots of *Gelsemium sempervirens* Ait., and in the leaves of *Bursaria spinosa* Cav. (8).

Herniarin has been isolated from leaves of *Herniaria hirsuta* L. (2), from lavender oil from *Lavandula officinalis* Chaix. (14), and from *Matricaria Chamomilla* L.

Imperatorin occurs in the root of *Imperatoria ostruthium* L. (20).

Limettin has been isolated from the rind of fruits of *Citrus* spp. (30).

Marmelosin is identical with *Imperatorin*. Isolated from fruit of *Aegle marmelos* Correa (8a).

Osthol occurs in the root of *Imperatoria ostruthium* L. (26).

Ostruthin occurs in the root of *Imperatoria ostruthium* L. (23).

Oxypeucedanin occurs in the root of *Imperatoria ostruthium* L. (22).

Peucedanin occurs in the root of *Peucedanum officinale* L. (21).

Scopoletin occurs in roots of *Avena sativa* L., *Gelsemium sempervirens* Ait. f., *Convolvulus scammonia* L., *Ipomoea Purga* Hayne, *Nicotiana Tabacum* L., *Scopolia japonica* Max.; in tubers of *Solanum tuberosum* L.; leaves of *Nicotiana Tabacum* L., *Fabiana imbricata* R. and P.; in shoots of *Atropa Belladonna* L.; in bark of *Prunus serotina* Ehrh.; in blossoms of *Chalcas exotica* (L.) Millsp.; in *Artemisia* spp. (9).

Skimmin has been isolated from *Skimmia japonica* Thumb. (24).

Umbelliferone occurs in the roots and rhizomes of various species of the Umbelliferae. Isolated from *Ferula Sumbul* Hook., *F. galbaniflua* Boiss. and Buhse, *F. Asafoetida* L., *F. Narthex* Boiss., *Imperatoria ostruthium* L., *Opopanax hispidum* Griseb., *Levisticum officinale* Koch., and from the bark of *Daphne Mezereum* L. (4).

Xanthotoxin occurs in the fruit of *Fagara xanthoxyloides* Lam. (25,28).

DISCUSSION

A compound must absorb the radiant energy with which it is irradiated before it can fluoresce, but absorption alone does not guarantee fluorescence. The compound must also have a resonating structure that can reradiate some of the absorbed energy as light instead of converting all of it into heat.

In the present investigation the 366 m μ mercury arc line was used to excite the fluorescence. Failure to observe fluorescence under our conditions may have been due to lack of absorption of the compound at

that wavelength (*e.g.*, 7-methylcoumarin²) or to lack of resonance, even though absorption actually occurred (*e.g.*, 7,8-dihydroxycoumarin and 5,7-dimethoxycoumarin, throughout the pH range; and 5,7-dihydroxycoumarin, 5,7-dihydroxy-4-methylcoumarin, and 6-methoxy-7-hydroxycoumarin² at pH 1.4). Furthermore, some of the compounds which are listed here as nonfluorescent may fluoresce when excited by other wavelengths.

It should also be noted that the shapes of the pH-fluorescence curves shown in the table may be modified by the spectral sensitivity of the photocell used in the fluorometer, by the transmission properties of the photocell filter, and by the chemical nature of the buffer solutions. The curves given here can be compared properly only with curves obtained with other instruments using selenium barrier-layer photocells, similar photocell filters, and similar buffer solutions. The foregoing remarks should be borne in mind when considering these data.

The fluorescence of several of the compounds tested increased with time of irradiation in the fluorometer. Usually the increase took place only in alkaline solution. This is due to conversion of the compound into a more fluorescent derivative. The fluorescence of 7-methoxycoumarin and 7-glucoxycoumarin increased upon irradiation at pH 11 and higher, the color of the fluorescence being bright blue. Coumarin, a nonfluorescent substance, when irradiated in alkaline solutions, rapidly formed a compound which exhibited a yellowish-green fluorescence and which has a pH-fluorescence curve nearly identical to that of a similarly treated solution of acetylcoumaric acid. This property has been made the basis of a quantitative method for coumarin assay in sweet clover (18,19). Several derivatives of coumarin (5-methyl-, 6-methyl-, 7-methyl-, 8-methyl- and 6-chlorocoumarin) also behave in this way. In this connection it is interesting to note the stabilizing effect of a methyl substitution in the 3- or 4- position on the molecule. Thus the 3-methyl-, 4-methyl-, 6-chloro-4-methyl-, and 7-chloro-4-methyl- derivatives do not decompose rapidly upon irradiation.

Nonfluorescent Derivatives

It can be seen from Table I that coumarin, 1-thiocoumarin, 2-thiocoumarin, coumarin-4-acetic acid, 3-acetylcoumarin, coumarin-3-car-

² The absorption spectra of these compounds were determined in the ultraviolet at several pH values with a Beckman DU spectrophotometer.

boxanilide, and all derivatives tested which have only methyl and chloro substituents are nonfluorescent. We have tested all six of the mono-methylcoumarins and 7-methylcoumarin with a saturated 4-carbon chain attached at the 3- and 4- positions (3-methyl-7,8,9,10-tetrahydrodibenzo- $[\beta,\delta]$ -pyrone). To these may probably be added the 4,7-dimethyl- and 3,4,7-trimethylcoumarins examined by Rangaswami and Seshadri (15).

It is among the hydroxy coumarins and their ethers that are found the most fluorescent derivatives, but of these quite a few do not exhibit fluorescence. In general, 8-substituted coumarin derivatives do not fluoresce. This statement applies to all the 8-substituted derivatives which we have tested, including daphnetin (7,8-dihydroxycoumarin) and its derivative with a 4-carbon chain attached at the 3- and 4- positions (3,4-dihydroxy-7,8,9,10-tetrahydrodibenzo- $[\beta,\delta]$ -pyrone), 7,8-dihydroxy-4-methylcoumarin, 7-hydroxy-8-acetyl-4-methylcoumarin, 8-methoxycoumarin, 8-methoxycoumarin-3-carboxylic acid, imperatorin, xanthotoxin, and osthol. Balaiah, Seshadri, and Venkateswarlu (1) report an absence of fluorescence from all ten of the 8-hydroxy- and 8-methoxy-derivatives which they studied, including 8-hydroxycoumarin, 8-methoxycoumarin-3-carboxylic acid, 7,8-dimethoxy-4-methylcoumarin, and 7,8-dimethoxy-4-methylcoumarin-3-ethylcarboxylate. Their compounds were dissolved in concentrated sulfuric acid, in alcohol and in dilute alkali (about pH 13) and were irradiated with sunlight. However, certain 8-methylcoumarins (*e.g.*, 7-hydroxy-8-methylcoumarin, 7-methoxy-8-methylcoumarin, and their 3-substituted derivatives) were reported as fluorescent, the color being more greenish than the unmethylated analogs (1,15).

We have also found 3-hydroxycoumarin, 4-hydroxycoumarin, 3,3'-methylene-bis-4-hydroxycoumarin (dicoumarol), 3,3'-ethyl carboxylate-methylene-bis-4-hydroxycoumarin, 4-methoxycoumarin, 5,7-dimethoxycoumarin, and oxypeucedanin to be virtually nonfluorescent. In addition, the above-mentioned investigators (1,15) report nine derivatives of 5-hydroxycoumarin, 5,7-dimethoxy-6-hydroxy-4-methylcoumarin and 5,6,7-trimethoxy-4-methylcoumarin as being nonfluorescent (1,15). It is possible, however, that some of these compounds might have exhibited fluorescence at some pH between -2 and 12.

One further group of nonfluorescent compounds is worthy of mention, the 3,4-dihydro derivatives. Umbelliferone (7-hydroxycoumarin) is one of the most fluorescent of all the coumarins; but 7-hydroxy-3,4-

dihydrocoumarin and three of its 4-substituted derivatives, as well as four 4-substituted 7-methoxy-3,4-dihydrocoumarins have been reported as nonfluorescent (1). This shows that the double bond in the pyrone ring is essential for the fluorogenous resonance of the molecule.

The Hydroxycoumarins

A discussion of the effect of position and number of hydroxyl substitutions on the fluorescence of coumarin may be found in the paper by Balaiah *et al.* (1). Umbelliferone (7-hydroxycoumarin) is by far the most fluorescent of the monohydroxy derivatives. The 3-hydroxy- and 4-hydroxycoumarins (see Table I) and the 5-hydroxy- and 8-hydroxy-

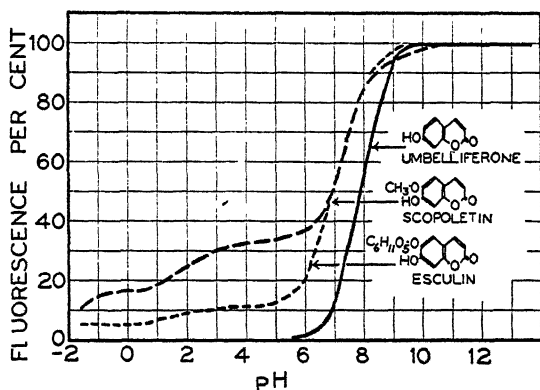


Fig. 1. The pH-fluorescence curves of esculin, scopoletin, and umbelliferone.

coumarins are nonfluorescent (1), and the 6-hydroxy-derivative is reported as exhibiting a weak greenish-blue fluorescence in acid solution, but not in dilute alkali or alcohol (1). The pH-fluorescence curve for umbelliferone makes a sharp drop between pH 8 and 9, decreasing to a low value at about pH 6. This curve is also characteristic of the following umbelliferone derivatives: 7-hydroxy-4-methylcoumarin, 7-hydroxy-5-methylcoumarin, 3-hydroxy-7,8,9,10-tetrahydribenzo- $[\beta, \delta]$ -pyrone, and ostruthin. The maximum fluorescence of these compounds is nearly the same as that of umbelliferone, with the exception of the 4-methyl-derivative, which is nearly twice as great.

The addition of a second hydroxyl group to umbelliferone at positions 5 or 6 causes at least a fifteenfold reduction in the maximum fluor-

escence and a drop in the pH-fluorescence curve in the range more alkaline than pH 8. The shape of the pH-fluorescence curve for 5,7-dihydroxycoumarin (Fig. 2) explains why it has been reported as non-fluorescent in acid and alkali (15). The addition of a second hydroxyl in the 8-position makes umbelliferone nonfluorescent.

The substitution of a methoxy or a glucoxy radical in the 6-position shifts the drop-off of the pH-fluorescence curve toward the acid and increases the fluorescence greatly in the pH range below 6 (Fig. 1).

The Methoxycoumarins

As might be expected, methoxy derivatives have very different fluorescent properties from their hydroxy analogs. The methoxyl is much

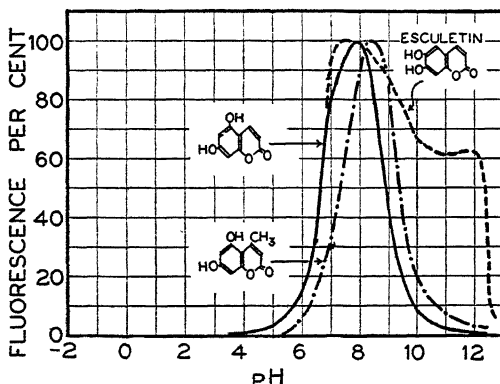


FIG. 2. The pH-fluorescence curves of esculletin, 5,7-dihydroxycoumarin, and 5,7-dihydroxy-4-methylcoumarin.

less effective than the hydroxyl group in influencing the fluorescence of the molecule. Thus 7-hydroxycoumarin is 300 times as fluorescent as 7-methoxycoumarin, and 5,7-dihydroxycoumarin is 200 times as fluorescent as 5,7-dimethoxycoumarin. Furthermore, where a hydroxyl added to the 6-position of 7-hydroxycoumarin reduces the maximum fluorescence by about 95%, a methoxyl in this position only reduces the fluorescence by about 25%.

Not only is the maximum fluorescence affected by methoxy groups, but the shapes of the pH-fluorescence curves are totally different. The 7-methoxycoumarin, a weakly-fluorescent compound, fluoresces in

strongly acid and strongly alkaline solutions with little fluorescence in the pH range from 1 to 11. The 6,7-dimethoxycoumarin behaves as an essentially neutral compound with a pH-fluorescence curve that is nearly flat in the pH range from -1 to 11.

The most fluorescent of the methoxy coumarins appears to be the 6,7-dimethoxy- derivative, followed by 7-methoxycoumarin. The 5,7-dimethoxy- and 8-methoxycoumarins are virtually nonfluorescent. The 5-methoxy-4-methyl- and 7-methoxy-4-methylcoumarins have been reported as feebly fluorescent; the 7,8-dimethoxy-4-methyl- and 5,6,7-trimethoxy-4-methylcoumarins, as nonfluorescent (1).

The 7-ethoxy- and 7-*n*-propyloxyderivatives behave in a similar manner to their 7-methoxy analogs, as would be expected.

TABLE II

*The Effect of Substitution in the 3-Position on the Fluorescence of
Certain Coumarin Derivatives*
Data from Table I

| | Maximum fluorescence at 1 μ mole/l. | | | | |
|-------------------------------|---|-------------------|---------------------|-----------|------------------|
| | Unsubstituted | 3-Carboxylic acid | 3-Ethyl carboxylate | 3-Acetyl- | 3-Phenyl- |
| Coumarin | <0.006 | 0.82 | 0.17 ^a | | |
| 7-Hydroxycoumarin | 380 280 | 376 | 334 302 | 88 | 398 ^b |
| 7-Methoxycoumarin | 1.26 | 166 | 118 | 0.82 | 202 ^a |
| 7- <i>n</i> -Propyloxcoumarin | 5.56 | 98 | | | |
| 6,7-Dimethoxycoumarin | 56 | 286 | 278 | | |
| 5,7-Dimethoxycoumarin | 0.046 | | 2.25 ^c | | 275 |

^a The 3-*n*-butylcarboxylate.

^b The 5-methyl- derivative.

^c The 6-hydroxy- derivative.

Substitution in the 3-Position

Substitution of a carboxyl group in the 3-position of a coumarin enhances the fluorescence of the derivative in the acid range, as can be seen from Table II. The shape of the pH-fluorescence curve of coumarin-3-carboxylic acid is characteristic and is retained in general by substituted 3-carboxylic acid coumarins. The esters of these compounds appear to be somewhat less fluorescent than their acids, and their pH-

fluorescence curves are nearly flat over most of the pH range, indicating that they are neutral compounds.

An acetyl group in the 3-position reduces the maximum fluorescence of the derivative (Table II) and changes the shape of the pH-fluorescence curve; while a phenyl group in this position greatly increases the maximum fluorescence (Table II) and also changes the shape of the pH-fluorescence curve.

Balaiah *et al.* (1) point out that carbonyl, cyano, and phenyl groups enhance the fluorescence of umbelliferone, when present in the 3-position, but not when present in the 4-position.

Uses for pH-Fluorescence Curves

Casparis and Manella (7) made an extensive study of various tests for the identification of seven natural coumarins (coumarin, umbelliferone, esculetin, ostruthin, bergapten, imperatorin, and peucedanin)

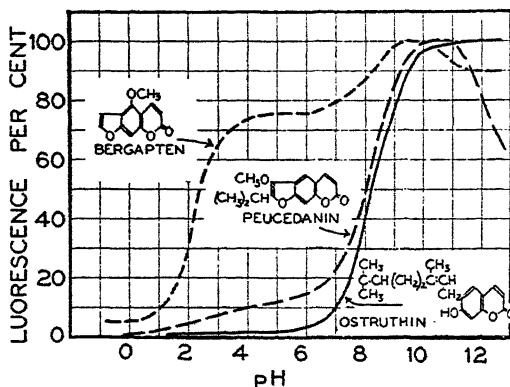


FIG. 3. The pH-fluorescence curves of bergapten, peucedanin, and ostruthin.

occurring in drugs. Among these tests were observations on the color of their fluorescence at several pH values. The pH-fluorescence curves should provide the pharmacologist with additional quantitative criteria for identifying these compounds (Figs. 1, 2, 3).

Leininger and Katz (12) describe a fluorometric method for the determination of malic acid and 2-naphthol, based upon the reaction of these two compounds in concentrated sulfuric acid. The fluorescent substance produced was thought to be 5,6-benzocoumarin. The pH-fluorescence

curves which we have made on the product of this reaction and on a sample of 5,6-benzocoumarin are identical. The curve of 5,6-benzocoumarin shows that the fluorescence should be measured at a pH above 1, if reproducible results are to be achieved without accurate pH control, but that considerable increase in sensitivity might be obtained by measuring very acid solutions (pH -2).

Another method for determining malic acid has been devised by Hummel (10). This is based upon the reaction of malic acid with orcinol to form 7-hydroxy-5-methylcoumarin. The fluorescence is measured in concentrated sulfuric acid, at a pH far below -2. Since 7-hydroxy-5-methylcoumarin exhibits relatively little fluorescence in the acid range, a test was made on this compound in concentrated sulfuric acid (94%). The fluorescence was greater under these conditions than at pH -1.6, but was only 27% of the maximum value obtained at pH 11.7. Hence, greater sensitivity for the above-mentioned malic acid method might be achieved by adjusting the pH to 10-12 before measuring the fluorescence.

Since these two methods for determining malic acid are about equally sensitive, the selection of the one to be used in a particular analysis should depend upon the nature of any fluorescent impurities. The determination should be made at a pH which will minimize the fluorescence of the contaminating substances.

The pH-fluorescence curve of scopoletin (7-hydroxy-6-methoxycoumarin) (Fig. 1) is sufficiently distinct to distinguish it from that of any of the other coumarins reported here, even 4-methylscopoletin. The curves for such closely related compounds as 7-hydroxy-5-methoxy-4-methylcoumarin, 7-hydroxy-6-glucoxycoumarin, 6,7-dihydroxycoumarin, 7-methoxy-6-hydroxy-4-methylcoumarin, 7-methoxy-5-hydroxy-4-methylcoumarin, and 6,7-dimethoxycoumarin are very different from that of scopoletin. This evidence lends support to the hypothesis that the coumarin derivative which was extracted from *Avena* roots and which had the same pH-fluorescence curve as scopoletin (9) was indeed scopoletin.

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Virgil Boekelheide of the University of Rochester; Dr. A. Butenandt of the University of Tübingen; Dr. Floyd DeEds of the U. S. D. A.; Dr. Ole Gisvold of the College of Pharmacy, University of Minnesota, for xanthotoxin; Dr. H. L. Haller of the U. S. D. A. for osthol; Dr. George H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, N. Y.; Dr. Donald Irish of the Dow Chemical Co., Midland, Mich.; Dr. J. H. Looker of Ohio State University; Mr. Humphrey Paget of the Wellcome Research Institution, London, England; Dr. George Rieveschl, Jr., of Parke, Davis and Co., Detroit, Mich.; Dr. Charles E. Sando of the U. S. D. A.; Dr. Hans Schmid of the University of Zürich; Dr. T. R. Seshadri of Delhi University, Delhi, India; Dr. Stanley Tarbell of the University of Rochester; Dr. H. Veldstra of N. V. Amsterdamsche Chininefabriek, Amsterdam, Holland; Dr. F. Wessely of the University of Vienna for the Späth preparations; Dr. M. M. Wolfrom of Ohio State University; Edwal Laboratories, Inc., Chicago, Ill.; Fritzsche Bros., New York; Koppers Co., Pittsburgh, Pa.; E. R. Squibb and Sons, New York; and Verona Chemical Co., Newark, N. J.

Without the generosity of the persons and companies listed above this investigation could not have been carried out. The study is a most interesting example of international coöperation.

SUMMARY

The color and relative intensity of the fluorescence of 98 coumarin derivatives are reported for hydrogen ion concentrations ranging from pH -1.6 to pH 12.6. The influence on fluorescence of hydroxyl, methoxyl, carboxyl, phenyl, methyl, and other substitutions at various positions on the coumarin molecule is discussed, and some of the uses of pH-fluorescence curves are briefly described. The distinctness of the pH-fluorescence curve of scopoletin (7-hydroxy-6-methoxycoumarin) from that of any other coumarin reported here supports the hypothesis that the coumarin derivative isolated from *Avena* roots was scopoletin.

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The Inactivation of Insulin By Tissue Extracts. V. The Effect of the Composition of the Diet on the Restoration of the Liver Insulinase Activity of the Fasted Rat ¹

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INTRODUCTION

In a previous communication (1) fasting was found to be associated with a marked reduction in the activity of the insulin-inactivating system (insulinase) of rat livers. It was also noted that the low insulinase activity of the fasted rat liver could be restored to the range of values obtained from the livers of unfasted rats by permitting the fasted animals to refeed *ad lib.* for a period of from 24–48 hr. on a balanced diet consisting of their normal laboratory rations. In an attempt to gather more information concerning the factors regulating the insulinase content of the liver, it was considered pertinent to compare the effects of a high-carbohydrate, a high-fat, and a balanced diet upon the restoration of the insulinase activity of rat liver following a period of fasting.

METHODS

Adult male rats (Sprague-Dawley strain) weighing from 211 g. to 334 g. and averaging 249 ± 25.42 g.³ were fasted for 72 hr., and were then divided into 3 groups each of which was refed with one of three different diets: (a) A balanced diet consisting of a stock laboratory ration (Rockland rat food pellets); (b) a high-carbohydrate diet; and (c) a high-fat diet. After the fast, each rat was given free access to one of the three

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² With the technical assistance of Gladys Perisutti and Jean Brand.

³ Mean \pm standard deviation.

diets for a period of 48 hr., after which time the animals were sacrificed for assay of their liver insulinase activity.

The high-fat and high-carbohydrate diets were prepared according to the specifications of Ingle (2).⁴ In the high-fat diet, 75% of the total caloric intake was supplied by fat, 5% by carbohydrate and 20% by protein; in the high-carbohydrate diet, 75% of the total caloric intake was supplied by carbohydrate, 20% by protein, and 5% by fat. Equal volumes of these diets were approximately isocaloric. The stock laboratory ration provided 58% of its calories in the form of carbohydrate, 30% by protein, and 12% by fat. The high-carbohydrate and high-fat diets were semifluid in consistency, whereas the stock laboratory ration was supplied in the form of pellets.

The insulinase activity of liver extracts prepared in a standardized manner was assayed according to a uniform procedure, the details of which have already been described (3). In this procedure, each milliliter of extract was incubated with 1 ml. of a solution containing 100 units of amorphous insulin⁵ for 30 min. at 37°C. At the end of this period, two 0.5-ml. aliquots of the reaction mixture were immediately diluted with water; one aliquot 1:35, the other 1:60.

It was our original intent to assay the residual insulin by determination of the blood sugar in a single sample drawn 50 min. after injection. The accuracy and adaptability of this procedure for the assay of relatively pure solutions of insulin has been established by Young and Romans (5) and by Pugsley and Rampton (6). During the course of the experiment, for reasons that will be discussed, it became evident that information concerning the preinjection, fasting blood sugar level would increase the precision of the assay. Accordingly, during most of the later phases of this investigation, blood sugar determinations were also performed on samples withdrawn just prior to the injections. The glucose content of all samples was determined by the Nelson modification of the Somogyi procedure (7).

The rabbits were injected according to a Latin-square design which permitted the use of a crossover type of schedule, the essential details of which are summarized in Table I. Although the use of a single crossover design involving injection of both dilutions would have permitted a factorial type of analysis, such a design was not utilized in view of the unpredictable differences in the changes in the insulin sensitivities of the rabbits (3) induced by successive injections of different dilutions of the incubation mixtures. Accordingly, animals which had once been injected with either one of the two dilutions were not subsequently

⁴ As was noted in a previous report (4), the caloric values in these diets differed from those prepared by Ingle.

⁵ We are indebted to the Eli Lilly Co. for generous supplies of a highly purified lot of amorphous insulin assaying 22.5 units/mg.

crossed with animals that had been injected with the other dilution. There were thus established two distinct series of crosses—one involving the 1:35 dilutions, the other involving the 1:60 dilutions. The three phases of each cross were performed on the same days of three successive weeks.

Inspection of Table I demonstrates that the design consisted essentially of two sets of five 3×3 Latin squares. The opportunity was thus

TABLE I
Design of Experiment

Roman numerals indicate each of three groups of rats. I represents group refed with stock rations. II indicates group refed with high-carbohydrate, and III the group refed with high-fat diets. On each of the indicated days of each week, one rat from each group was sacrificed and the liver extracts prepared from the respective rats were injected into groups of rabbits according to the sequence indicated.

| 1:35 dilutions | | | | | | | | | | | | | | | | |
|----------------------|-----|--------|-----|-----|---------|-----|-----|-----------|-----|-----|----------|-----|-----|--------|-----|-----|
| Day of week injected | | Monday | | | Tuesday | | | Wednesday | | | Thursday | | | Friday | | |
| Rabbit group | | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
| Week of experiment | 1st | I | II | III | I | II | III | I | II | III | I | II | III | I | II | III |
| | 2nd | III | I | II | II | III | I | III | I | II | II | III | I | III | I | II |
| | 3rd | II | III | I | III | I | II | II | III | I | III | I | II | II | III | I |
| 1:60 dilutions | | | | | | | | | | | | | | | | |
| Rabbit group | | A' | B' | C' | D' | E' | F' | G' | H' | I' | J' | K' | L' | M' | N' | O' |
| Week of experiment | 1st | I | II | III | I | II | III | I | II | III | I | II | III | I | II | III |
| | 2nd | II | III | I | III | I | II | II | III | I | III | I | II | II | III | I |
| | 3rd | III | I | II | II | III | I | III | I | II | II | III | I | III | I | II |

provided to estimate the effect of the refeeding of each of the three diets on the liver insulinase activities in 15 rats in each group. Since the opportunities for randomization in a 3×3 Latin square are restricted, it was considered more desirable to minimize the influence, on the changes in insulin sensitivity, of the injection of the preceding type of extract by an alternation between the two possible combinations of progression of injections on the odd and even days. This aspect of the design is also illustrated in Table I.

RESULTS

The 72-hr. period of fasting resulted in similar weight losses in each of the 3 groups of 15 rats. Furthermore, the gains in weight during the 48-hr. period of refeeding following the fast were similar, regardless of the type of diet ingested. These data, which are summarized in Table II, would appear to indicate that, irrespective of the widely different compositions and consistencies of the three different diets, each was consumed in approximately equicaloric amounts.

On the other hand, the various dietary treatments resulted in some differences in the three groups of rats. Those animals fed high-carbohydrate and high-fat diets were found to possess livers visibly paler in

TABLE II
Effect of Type of Diet on Rats^a
Each diet fed *ad lib.* for 48 hr. following a 72-hr. fast

| | Stock laboratory rations | High-carbohydrate diet | High-fat diet |
|--|--------------------------|----------------------------|---------------|
| Number of rats | 15 | 15 | 15 |
| Final body weight, g. | 223±1403 | 230±30.15 | 231±28.52 |
| Change in body weight, ^b g. | -20±8.61 | -24±5.61 | -20±8.70 |
| Liver weight, g. | 8.6±0.79 | 11.7±1.17 ^c | 9.4±1.41 |
| Liver weight/body weight | 0.0385±0.0032 | 0.0512±0.0062 ^c | 0.0409±0.0051 |
| Nitrogen content of liver extracts, % | 0.706±0.043 | 0.56±0.051 ^c | 0.715±0.044 |

^a All values stated in terms of mean ± standard deviation.

^b Total change from start of fast to end of refeeding period.

^c Difference from other types of treatment is highly significant (p. < 0.001).

appearance than those refed with the normal stock laboratory ration; this paleness was most apparent in the animals fed the high-fat diet. The weights of the livers obtained from the rats fed a high-carbohydrate diet were significantly heavier than those of the animals fed a high-fat or the stock pellet diet. The ratio: liver weight/body weight was also significantly higher in the group of rats refed with a high-carbohydrate diet. Finally, the percentage of nitrogen in the extracts obtained from the livers of the rats refed with a high-carbohydrate diet was significantly lower than the percentage of nitrogen in the extracts obtained from the pellet-refed and the fat-refed rats. However, in view of the

TABLE III

Blood Sugars (mg.-%) in Rabbits Injected with Reaction Mixtures Prepared from Livers of Rats

The upper set of figures in each row refers to the fasting, preinjection levels; the lower set of figures represent the blood sugar levels in the 50-min. samples. The type of refed rat whose liver insulinase activities were estimated by the above results detailed in Table I.

| Rabbit group | | 1:35 dilutions | | | | | | | | | | | |
|----------------------------------|------|-----------------|----|----|-----|----|----|-----|-----|----|-----|----|----|
| | | A | | | B | | | C | | | D | | |
| Individual rabbits in each group | Week | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| | | 52 | 63 | 47 | 49 | 64 | 51 | 28 | 38 | 20 | 57 | 08 | 76 |
| of | 1st | 75 | 77 | 72 | 75 | 77 | 86 | 65 | 100 | 70 | 88 | 96 | 66 |
| | 2nd | 30 | 50 | 42 | 56 | 50 | 56 | 45 | 73 | 30 | 23 | 54 | 35 |
| experiment | 3rd | 76 | 65 | 78 | 70 | 67 | 61 | 80 | 68 | 70 | 79 | 80 | 74 |
| | | 28 | 28 | 42 | 40 | 42 | 31 | 53 | 34 | 28 | 40 | 40 | 34 |
| | | 1:60 dilutions | | | | | | | | | | | |
| Rabbit group | | A' | | | B' | | | C' | | | D' | | |
| Individual rabbits in each group | Week | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| | | 64 | 80 | 60 | 46 | 65 | 31 | 55 | 55 | 69 | 65 | 47 | 87 |
| of | 1st | 73 | 73 | 61 | 109 | 86 | 88 | 82 | 74 | 93 | 73 | 85 | 66 |
| | 2nd | 40 | 63 | 50 | 73 | 65 | 54 | 51 | 51 | 57 | 30 | 43 | 38 |
| experiment | 3rd | 65 | 70 | 59 | 65 | 71 | 71 | 58 | 47 | 63 | 64 | 89 | 60 |
| | | 36 | 35 | 47 | 41 | 37 | 47 | 29 | 27 | 58 | 45 | 58 | 43 |
| | | 1:100 dilutions | | | | | | | | | | | |
| Rabbit group | | A'' | | | B'' | | | C'' | | | D'' | | |
| Individual rabbits in each group | Week | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| | | 64 | 80 | 60 | 46 | 65 | 31 | 55 | 55 | 69 | 65 | 47 | 87 |
| of | 1st | 73 | 73 | 61 | 109 | 86 | 88 | 82 | 74 | 93 | 73 | 85 | 66 |
| | 2nd | 40 | 63 | 50 | 73 | 65 | 54 | 51 | 51 | 57 | 30 | 43 | 38 |
| experiment | 3rd | 65 | 70 | 59 | 65 | 71 | 71 | 58 | 47 | 63 | 64 | 89 | 60 |
| | | 36 | 35 | 47 | 41 | 37 | 47 | 29 | 27 | 58 | 45 | 58 | 43 |

TABLE III—Continued

| 1:35 dilutions | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------------------------------|----------------------------------|----|----|-----|-----|----|----|-----|-----|----|----|----|----|-----|-----|-----|-----|-----|----|----|-----|-----|-----|----|-----|
| Rabbit group | | H | | I | | J | | K | | L | | M | | N | | O | | | | | | | | | |
| Individual rabbits in each group | Week of experiment | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | | | | | | |
| | | 88 | 88 | 104 | 92 | 86 | 91 | 109 | 100 | 93 | 95 | 96 | 96 | 95 | 112 | 106 | 101 | 102 | 92 | 96 | 101 | 125 | 124 | | |
| | | 67 | 36 | 48 | 31 | 50 | 61 | 95 | 96 | 77 | 66 | 64 | 91 | 42 | 89 | 52 | 60 | 66 | 80 | 64 | 73 | 74 | 70 | 68 | 59 |
| | | 94 | 88 | 89 | 142 | 80 | 65 | 92 | 78 | 81 | 95 | 73 | 87 | 105 | 103 | 87 | 80 | 87 | 73 | 86 | 97 | 93 | 73 | 64 | 103 |
| | | 56 | 44 | 51 | 89 | 53 | 28 | 44 | 56 | 40 | 57 | 55 | 66 | 58 | 66 | 45 | 41 | 38 | 29 | 37 | 42 | 54 | 64 | 42 | 67 |
| | | 82 | 94 | 91 | 65 | 83 | 65 | 89 | 87 | 68 | 81 | 82 | 79 | 89 | 101 | 77 | 74 | 76 | 59 | 44 | 77 | 66 | 70 | 65 | 82 |
| | | 50 | 47 | 59 | 27 | 28 | 18 | 20 | 51 | 35 | 30 | 45 | 26 | 36 | 51 | 45 | 20 | 47 | 34 | 35 | 18 | 31 | 37 | 30 | 36 |

| 1:60 dilutions | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------------------------------|----------------------------------|-----|----|-----|-----|----|----|----|-----|-----|-----|-----|----|----|-----|-----|-----|----|----|-----|-----|-----|-----|-----|-----|
| Rabbit group | | H' | | I' | | J' | | K' | | L' | | M' | | N' | | O' | | | | | | | | | |
| Individual rabbits in each group | Week of experiment | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | | | | | | |
| | | 102 | 82 | 85 | 115 | 99 | 89 | 76 | 109 | 105 | 107 | 101 | 85 | 89 | 111 | 122 | 126 | 81 | 92 | 102 | 107 | 114 | 150 | 116 | 128 |
| | | 58 | 94 | 56 | 55 | 44 | 53 | 91 | 74 | 82 | 95 | 95 | 72 | 76 | 96 | 100 | 80 | 81 | 75 | 71 | 84 | 62 | 84 | 68 | 87 |
| | | 73 | 91 | 101 | 79 | 87 | 93 | 84 | 64 | 87 | 86 | 89 | 84 | 75 | 81 | 98 | 73 | 70 | 87 | 123 | 89 | 71 | 90 | 91 | 74 |
| | | 52 | 57 | 40 | 43 | 39 | 64 | 52 | 63 | 104 | 64 | 56 | 52 | 40 | 37 | 60 | 42 | 24 | 48 | 62 | 81 | 31 | 77 | 48 | 44 |
| | | 64 | 63 | 65 | 69 | 51 | 82 | 72 | 83 | 104 | 83 | 82 | 92 | 59 | 82 | 87 | 62 | 82 | 88 | 71 | 87 | 77 | 73 | 79 | 65 |
| | | 23 | 23 | 18 | 59 | 35 | 51 | 62 | 81 | 101 | 58 | 44 | 58 | 32 | 49 | 58 | 36 | 29 | 44 | 31 | 60 | 83 | 41 | 51 | 37 |

larger weights of the livers of the carbohydrate-refed rats, the absolute quantity of nitrogen in the total volume of extracts obtained from the three groups of animals was similar. These findings are also summarized in Table II.

Thirty groups of five rabbits each were planned for use for estimation of the insulinase activities according to the procedure outlined above. Fifteen of these groups were injected with 1:35 dilutions of the reaction mixtures and 15 with 1:60 dilutions. For reasons discussed more completely in another paper (3), not all of the rabbits survived the 3-week crossover design. Previously unused rabbits were substituted, whenever possible, for those who failed to survive. Of the 150 rabbits that received injections for the first time during the 1st week of experiment, a total of only 112 received the entire progression of scheduled injections. Of these, 53 survived the entire crossover with the 1:35 dilutions and 59 survived the progression of injections with the 1:60 dilutions.

In order to condense the presentation of the data, the results of the blood sugar determinations in Table III are confined to the first three rabbits in each group that survived the entire 3-week crossover. Consideration of the remainder of the data did not influence, to any degree, the conclusions derived below. As was noted above, values for the preinjection blood sugar levels were lacking in the first few groups of rabbits.

As has been the custom in our previous work with the determination of insulinase activity, no attempt has been made to express such activities in terms of the absolute amount of insulin destroyed. It was considered more pertinent merely to compare the relative activities of the liver extracts of the three groups of rats in terms of the same index. For this purpose, we have utilized each of three different methods of expression of the figures obtained from analysis of the 50-min. samples: (a) blood sugar level in actual mg.-%; (b) mg.-% drop from the fasting level; (c) percentage of the fasting level. In deference to brevity the results will be presented only in terms of the actual mg.-% blood sugar. However, analysis of the results by either of the other two modes of expression did not alter the conclusions in any respect whatsoever.

The significance of the data was estimated by the conventional method of an analysis of variance adapted to a Latin-square design (8). For this purpose, all five of the 3×3 Latin squares were available for analysis in terms of the actual mg.-% blood sugar level at 50 min. Since fasting blood sugar levels were lacking in the case of the first few groups

of rabbits, analysis in terms of either mg.-% drop or percentage of the initial level could be performed only in the case of the last three of the Latin squares. In other words, fifteen replicates of rats were available for analysis in terms of the 50-min. blood sugar level; nine replicates were available for analysis in terms of the other two methods of expression of the results.

The failure of many of the animals to survive further complicated the analysis since the different groups contained a varying number of animals that received all three injections of the cross. Due to the profound changes in insulin sensitivities of the rabbits induced by the progressive injections of the incubation mixtures (3), it was not deemed justifiable to supply missing data with the results obtained from the injection of substituted, previously unused rabbits. However, it may be noted that inclusion of such results in the analyses failed to alter the significance of any of the conclusions.

A minimum of three rabbits survived in each of the fifteen groups. The mean value obtained from the first three surviving animals in each group has been utilized in the analysis of the effect of the dietary treatments on the insulinase activity of the rat liver extracts. It should be noted however, that the use of various other combinations of animals produced only insignificant changes in the different estimates of variance. Thus, the use of the values obtained from either the first surviving rabbit of the group or from the weighted or unweighted mean of all of the surviving rabbits of each group gave results identical in their significance with those to be presented. This was true, regardless also of whether the data from all five Latin squares (in those instances where such results were available) were utilized in the analysis, or where the data from only the last four or last three of the Latin squares were analyzed.

In every instance no significant differences could be found in the insulinase activities of the liver extracts obtained from the three groups of rats refed with each of the three different diets whose effect was examined. To illustrate the lack of effect of the diet on the restoration of insulinase activity, the results of a typical analysis of variance are listed in Table IV. It will be noted that the variance due to the differences produced by the three dietary treatments was less than the residual error of the experiment. This relatively insignificant variance was noted in every one of the types of analysis described above.

The most profound change noted, in every instance, was the significant increase in the insulin sensitivities of the rabbits during the three successive weeks of the cross. This source of variance has been noted and described more fully in another report (3). Associated with this progressive increase in insulin sensitivity of the rabbits was a progressive decrease in their fasting blood sugar, a phenomenon also previously described. Since the estimate of the insulinase activity might have been influenced by the concomitant variation in the fasting blood sugars, it was considered advisable to adjust for these changes by an analysis of covariance (9). This type of analysis, a sample of which is illustrated

TABLE IV

Analysis of Variance of Estimates of Insulinase Activities

All insulinase activities expressed in terms of mg.-% blood sugars in 50-min. samples. The data used for these calculations are the mean values of the 3 rabbits of each of the 15 groups in Table III.

| Source of variation | Degrees of freedom | 1:35 dilutions Mean square | 1:60 dilutions Mean square |
|----------------------|--------------------|-------------------------------|-------------------------------|
| Weeks ^c | 2 | 2,260.83 ^a | 1,719.75 ^a |
| Rabbits ^d | 14 | 90.64 | 298.14 ^b |
| Diets ^e | 2 | 30.43 | 43.09 |
| Error | 26 | 117.78 | 129.30 |

^a $p < 0.001$.

^b $p < 0.05$.

^c Refers to the variability due to progressive changes in the insulin sensitivities of the rabbits during the 3 successive weeks of the cross.

^d Indicates the degree of variability among different rabbits.

^e Indicates the variance in insulinase activities attributable to the three different diets given the groups of rats.

in Table V, failed to alter the significance of the previous conclusions, except to change slightly, in certain instances, the variances attributable to the rabbits and the 3 weeks of the cross. When all of the available data were analyzed, the significance of the variances attributable to "weeks" was not affected.

DISCUSSION

In a series of earlier observations on the effect of the composition of the diet during the period of refeeding on the restoration of insulinase activity of rat livers (10), results were obtained which appeared to indi-

cate a greater degree of restoration of activity in those rats refed with a high-carbohydrate diet. Subsequently it was possible to demonstrate that this apparent effect was due to the above described changes in insulin sensitivity in the rabbits induced as the result of repeated injections of liver extracts. The data reported in this paper were derived from a series of experiments which were designed to permit a precise estimate of the component sources of variability. As a result, it now appears permissible to draw valid conclusions concerning the effect of the three diets on the liver insulinase activity.

It has been previously demonstrated (1) and repeatedly confirmed in many subsequent observations, that the insulinase activity of rat liver extracts decreased markedly during a 72-hr. period of fasting and that

TABLE V

Analysis of Covariance of Estimates of Insulinase Activities

All insulinase activities expressed as in Table IV. The data used for the following calculations, in which the values of the 50-min. samples have been adjusted for the variations in the preinjection blood sugar levels, are derived from the mean values of the 3 rabbits of each of the last 9 groups of rabbits in Table III.

| Source of variation | Degrees of freedom | 1:35 dilutions Adjusted mean square | 1:60 dilutions Adjusted mean square |
|---------------------|--------------------|--|--|
| Weeks | 2 | 385.42 | 912.05 ^a |
| Rabbits | 8 | 49.01 | 380.21 ^b |
| Diets | 2 | 69.81 | 5.24 |
| Error | 13 | 116.22 | 127.99 |

^a $p < 0.01$.

^b $p < 0.05$.

this activity was restored to normal levels by refeeding such animals with their stock laboratory rations. The data reported in this paper indicate that the restoration of the insulinase activity per unit weight of liver in previously fasted rats occurred equally well with the high-carbohydrate, the high-fat, and the stock diets. Such findings would appear to indicate that approximately equivalent amounts of the dietary factors, responsible for the maintenance of liver insulinase activity in the unfasted rat (4) and for the restoration of the diminished activity in the fasted rat, were present in each of the three diets. It appears improbable that the total caloric intake is the determining factor since previous observations failed to demonstrate a difference in the liver

insulinase activities in rats fed a normal and very high caloric diet (4). Since all three diets contain an adequate amount of protein, it is possible that it is this component which is the important determinant in the maintenance and restoration of liver insulinase.

On the other hand, it must be emphasized that these calculations compare insulinase contents per unit weight of liver. Since the rats refed with the high-carbohydrate diet were found to possess significantly heavier livers than those in the other two groups, it would appear that the former group of rats contain more total liver insulinase than the latter. Furthermore, since the ratio: liver weight/body weight was also highest in the rats refed with the high-carbohydrate diet, such rats contain more liver insulinase per unit body weight.

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SUMMARY AND CONCLUSIONS

1. An experiment was designed to permit estimation of the effect of three different diets on the restoration of liver insulinase activity in previously fasted rats.

2. During a 48-hr. period of refeeding following a 72-hr. fast, the insulinase activity per unit weight of liver was restored equally well by a high-carbohydrate, a high-fat and a balanced stock diet.

3. The high-carbohydrate diet produced a greater increase in the total insulinase activity of the liver than did the high-fat or balanced stock diets.

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An Improved Method of Hydrolysis for Use in the Microbiological Determination of Tryptophan in Human Milk

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INTRODUCTION

Determination of tryptophan has always been largely a problem of hydrolysis and although many methods have been employed in analyses of animal and plant proteins for tryptophan, data concerning the hydrolysis of whole human milk are not available. Plimmer and Lowndes (1) hydrolyzed milk proteins with $\text{Ba}(\text{OH})_2$; Beach *et al.* (2) and Block and Bolling (3) employed NaOH , but several authors (4-7) have pointed out the disadvantages encountered with each method. Hydrolysis procedures which are suitable for the determination of tryptophan by chemical methods may not be suitable for microbiological assay and this report presents results, obtained with various hydrolysis procedures, indicating the preferable method of hydrolysis for the microbiological determination of tryptophan in whole human milk.

EXPERIMENTAL

Tryptophan was determined after various methods of hydrolysis (7, 9, 10) in samples of dried, whole human milk obtained from women at various intervals in lactation (8) and in samples of commercial, vitamin-free cow casein.¹ Aliquots of 0.25 g. of the same sample were weighed into test tubes, glass beakers, and nickel crucibles. Test tubes were sealed, beakers were covered with larger beakers, and the nickel crucibles were covered with nickel lids. Some aliquots were treated with 8 ml. of 4 *N* NaOH , others with 4 ml. of 6 *N* $\text{Ba}(\text{OH})_2$, and other groups with either of these alkalis plus cysteine. Whenever $\text{Ba}(\text{OH})_2$ was used, 2.1 g. of anhydrous $\text{Ba}(\text{OH})_2$ and 4 ml. of distilled water were added to each sample. The hot NaOH -cysteine solution was prepared

¹ The Borden Co., Labco brand.

according to Kuiken *et al.* (7), using 50 mg. of L-cysteine hydrochloride with 8 ml. of 4 *N* NaOH in a 50-ml. pyrex beaker; this was covered with a larger beaker and autoclaved 1 hr. at 15 lb. pressure. While the solution was still hot, an additional 50 mg. of cysteine and the sample were added. Hydrolysis with solutions of NaOH and cysteine which had not been preheated also was tested.

Autoclaving periods ranging from 10 to 16 hr. at 15 lb. pressure and 120° were used. After autoclaving, the pH of each hydrolysate was adjusted to 4.6, using HCl for NaOH digests and H₂SO₄ for Ba(OH)₂ digests. "Hy-flow supercell"² was added to each hydrolysate before filtration with suction through Whatman filter paper No. 42. After thoroughly washing a precipitate, the filtrate was transferred to a 100-ml. volumetric flask, the pH of 4.6 was checked, the solution was brought to volume, then refiltered into 125-ml. glass-stoppered Erlenmeyer flasks. If not assayed immediately, hydrolysates were covered with a layer of benzene and refrigerated.

The tryptophan liberated by the various methods of hydrolysis was determined by microbiological assay, using the media and technique described in another paper (11). Initial assays were done with the test organisms *Leuconostoc mesenteroides* and *Streptococcus faecalis*. *Lactobacillus arabinosus* was not used because it responds to indole and anthranilic acid. The growth of *Leuc. mesenteroides* was found to be inhibited by the presence of various concentrations of salt and thereafter only *S. faecalis* was used.

RESULTS AND DISCUSSION

The amounts of tryptophan liberated from aliquots of one human milk sample by ten different hydrolytic procedures (Table I), varied from 1.55 to 2.12 mg./g. of dried whole milk.

Aliquots hydrolyzed in nickel crucibles with NaOH, whether protected against oxidation by addition of cysteine or not, gave lower values than did hydrolysates in glass containers. Of the NaOH hydrolysates in nickel crucibles, those prepared with unheated NaOH-cysteine showed the largest amounts of tryptophan; of those in glass containers, preparations with the unheated solution showed the lowest contents, the mean (Table I) being only slightly higher than the mean of the identical preparations in nickel crucibles. The hydrolysates with NaOH

² Johns-Manville and Co.

only, and with hot NaOH-cysteine, in glass containers, gave the highest and almost the same yields of tryptophan. Values for $\text{Ba}(\text{OH})_2$ hydrolysates were higher than those for NaOH hydrolysates prepared under the same conditions, with greater yields from the aliquots in nickel crucibles. Experimentation showed that following hydrolysis with $\text{Ba}(\text{OH})_2$ in nickel crucibles, results obtained after autoclaving for 11 hr. were as good, or better, as those found after samples were autoclaved 16 hr.

To compare the hydrolysis methods which gave the highest values, Table II presents the results from determinations of tryptophan in hydrolysates of 14 different samples of human milk and 8 aliquots of

TABLE I
*Tryptophan Liberated From 10 Identical Aliquots of Human Milk
by Different Hydrolytic Procedures^a*
Values in mg./g. dried whole milk

| Hydrolyzing agent | Container | |
|---|-----------|--------|
| | Glass | Nickel |
| 4 N NaOH | 1.94 | 1.60 |
| 4 N NaOH + cysteine, hot ^b | 1.95 | 1.55 |
| 4 N NaOH + cysteine, unheated | 1.70 | 1.62 |
| 6 N $\text{Ba}(\text{OH})_2$ + cysteine, unheated | 1.80 | 1.84 |
| 6 N $\text{Ba}(\text{OH})_2$ | 2.00 | 2.12 |

^a All aliquots were autoclaved 16 hr. and each value represents 2 or more assays. Nitrogen content: 23.21 mg./g. dry weight.

^b As described by Kuiken *et al.* (7).

casein, prepared in glass beakers with hot NaOH + cysteine (7), and in corresponding hydrolysates prepared in nickel crucibles with $\text{Ba}(\text{OH})_2$. The difference between results for hydrolysates prepared by the two methods was found to be highly significant at a 1% level by the *F* test, subsequent to an analysis of variance (12). For 12 of the 14 milk samples, tryptophan values were 0.04–0.63 mg. higher for the $\text{Ba}(\text{OH})_2$ hydrolysates: for 2 samples, the $\text{Ba}(\text{OH})_2$ hydrolysates were lower by negligible amounts, 0.06 and 0.09 mg. The tryptophan values for hydrolysates of casein aliquots prepared by the 2 methods were significantly different, but values for hydrolysates prepared by the same method did not differ significantly, indicating a relatively high degree of reproducibility for the microbiological method of tryptophan determination.

Nine $\text{Ba}(\text{OH})_2$ hydrolysates of human milk assayed before and after refrigeration for 36 days did not show a significant difference between the tryptophan contents before and after storage. A test mixture of chemically pure amino acids, vitamins, and salts was assayed with each group of samples. Of these assays, only one failed to yield tryptophan values within $\pm 3\%$ of the known content. Duplicate milk and casein samples checked within $\pm 5\%$.

TABLE II

Tryptophan in 14 Human Milk Samples and 8 Aliquots of Cow Casein
Values in mg./g. dry material

| Human milk ^a | | | Casein ^b | |
|-------------------------|--|---|--|---|
| Nitrogen | Hot NaOH-cysteine hydrolysate ^c | $\text{Ba}(\text{OH})_2$ hydrolysate ^d | Hot NaOH-cysteine hydrolysate ^c | $\text{Ba}(\text{OH})_2$ hydrolysate ^d |
| 14.73 | 1.21 | 1.35 | 12.4 | 13.3 |
| 16.10 | 1.49 | 1.89 | 11.5 | 13.6 |
| 19.06 | 1.58 | 1.90 | 11.9 | 13.9 |
| 22.81 | 1.77 | 2.22 | 11.2 | 12.8 |
| 15.53 | 1.29 | 1.56 | 12.5 | 13.2 |
| 14.81 | 1.28 | 1.44 | 11.1 | 12.4 |
| 25.35 | 2.29 | 2.60 | 11.3 | 13.7 |
| 15.81 | 1.65 | 1.59 | 12.0 | 13.7 |
| 15.91 | 1.57 | 1.61 | | |
| 11.91 | 1.10 | 1.31 | | |
| 14.02 | 1.25 | 1.16 | | |
| — | 1.89 | 2.52 | | |
| — | 2.34 | 2.55 | | |
| 16.02 | 1.56 | 1.69 | | |

^a Each value represents 2 or more assays.

^b Corrected for ash and moisture. Nitrogen content 1:48.92 mg./g.

^c Kuiken *et al.* (7).

^d Prepared in nickel crucible, autoclaved 11 hr.

Recovery experiments with DL-tryptophan hydrolyzed by various methods indicated that when hydrolyzed alone tryptophan is much more susceptible to destruction than it is when hydrolyzed in the presence of other amino acids or proteins. In the presence of gelatin, the hot NaOH + cysteine hydrolysis resulted in recovery of 91% of the tryptophan, but $\text{Ba}(\text{OH})_2$ hydrolysis yielded 97%.

The use of $\text{Ba}(\text{OH})_2$ as a hydrolytic agent has been criticized because of possible absorption of tryptophan on the BaSO_4 precipitate. Chemical and microbiological assays of these precipitates did not provide evidence of the presence of tryptophan. The $\text{Ba}(\text{OH})_2$ hydrolysis of human milk in nickel crucibles for tryptophan assay avoids: the problems presented by silica gels which form as the result of reaction with glass; the probable tryptophan destruction which seems to occur in hydrolysis with NaOH ; and the addition of protective agents. This method of hydrolysis provides clear, colorless hydrolysates, and with it the tryptophan values obtained are higher than those procured by other hydrolysis methods. As Greenhut *et al.* (4) pointed out, it seems logical to select for analytical purposes the procedure which produces the greatest yields.

Since completion of this experimental work, data obtained by hydrolysis with NaOH in stainless steel beakers has been published (13), and the authors suggest the use of nickel beakers as substitutes for those of stainless steel.

SUMMARY

In aliquots of human milk, tryptophan was determined by microbiological assay following hydrolysis with NaOH and $\text{Ba}(\text{OH})_2$ in nickel and in glass containers. Values were greater with $\text{Ba}(\text{OH})_2$ than with NaOH and addition of cysteine did not produce greater yields of tryptophan. Hydrolysates prepared with $\text{Ba}(\text{OH})_2$ in nickel crucibles contained larger amounts of tryptophan than those hydrolyzed in glass beakers, and assays of aliquots of casein hydrolyzed under these conditions showed a relatively high degree of reproducibility for the method.

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Studies on the Proteins and Lipides of Plasma Fractions of X-Ray-Irradiated Rats¹

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INTRODUCTION

The information concerning the effect of total body X-radiation on the plasma proteins is limited. In a recent paper (1), electrophoretic analyses of whole plasma of X-radiated dogs showed that protein distribution is not altered appreciably until shortly before death. Previous investigations (2,3) have indicated that electrophoretic and chemical analyses of whole plasma of rats yield a limited amount of information as compared with that obtainable after plasma protein fractionation. This paper is concerned with the effect of total body X-radiation of rats on the electrophoretic patterns and on the nitrogen and lipide composition of four plasma fractions.

METHODS

Inbred male rats of Wistar stock, 60-70 days old and weighing between 150-200 g. were used as experimental animals. The animals were maintained on a diet of Gaines dog meal and Purina checkers. Food was withdrawn the night before the animals were sacrificed; water was always available. The animals received total body X-radiation in a single exposure at dose levels of 500, 600, 750, 1000, 1500, 1750, and 2000 röntgens (r). During irradiation the rats were placed in a box subdivided into 8 stalls; the cover, made of Plexiglass, contained a large number of holes for ventilation. The box was surrounded on the sides and bottom with a thick layer of masonite to give maximum back scatter and uniform radiation to all animals. The following radiation factors were employed: 250 kv., 15 ma., target to subject distance 100 cm., no filters, half value layer 0.5 mm. Cu. The LD 50 dose, according to these procedures, is between 600 and 700 r at moderate temperatures. Groups of animals were sacrificed at varying

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² With the technical assistance of Jasper P. Lewis.

intervals after irradiation. The rats were anesthetized with sodium pentobarbital and were exsanguinated by drawing blood from the aorta into a syringe moistened with heparin. The plasmas of each group were pooled and prepared for fractionation analysis.

In a previous paper (2) directions are given for fractionating rat plasma. These procedures have been slightly modified as follows: (a) the plasma is diluted 5 times with distilled water and Fraction I is precipitated as usual; (b) Fraction II + III is precipitated from Super I at 19% ethanol, pH 6.5, $\Gamma/2$, 0.026 at -5° ; Fraction IV-4 is precipitated from Super II + III at 19% ethanol, pH 5.2, $\Gamma/2$, 0.026 and -5° ; Fraction V is obtained from Super IV-4 as previously described.

The plasma and fraction solutions were dialyzed against a veronal buffer ($\Gamma/2$, 0.1) at pH 8.6 for several days in the cold room. Electrophoresis was carried out in the Tiselius apparatus at 2° , the patterns being recorded by the scanning method of Longworth. Both micro and macro cells with capacities of 2 and 11 ml. were used. Mobility calculations were based on the distance from the peak of salt effect to the peak of the respective components. The areas of the ascending patterns were measured.

The total lipides of the plasma and fractions were extracted with hot acetone-absolute ethanol (1:1); aliquots were analyzed for cholesterol according to the pro-

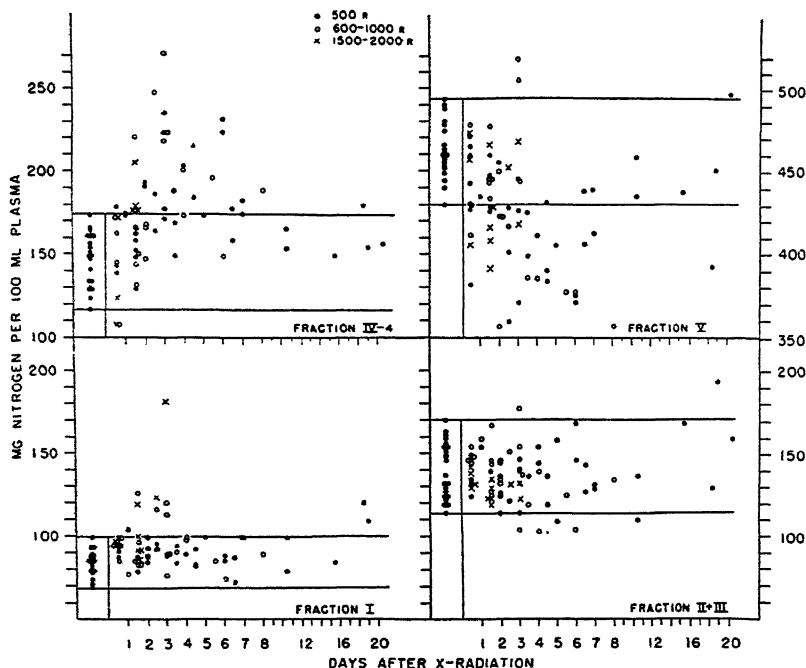


Fig. 1. Protein nitrogen contents of four rat plasma fractions. The maximum and minimum values for controls are designated by the two parallel lines in this and in subsequent figures.

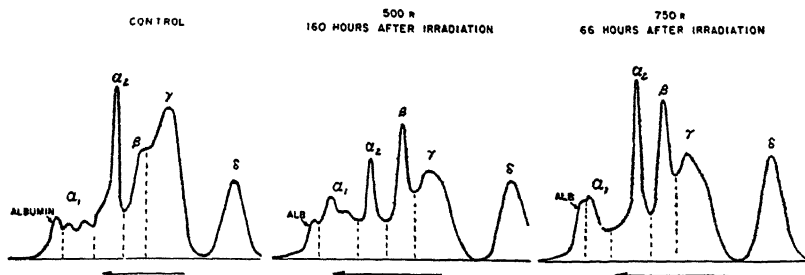


FIG. 2. Electrophoretic patterns of Fraction II + III of control and X-irradiated rats.

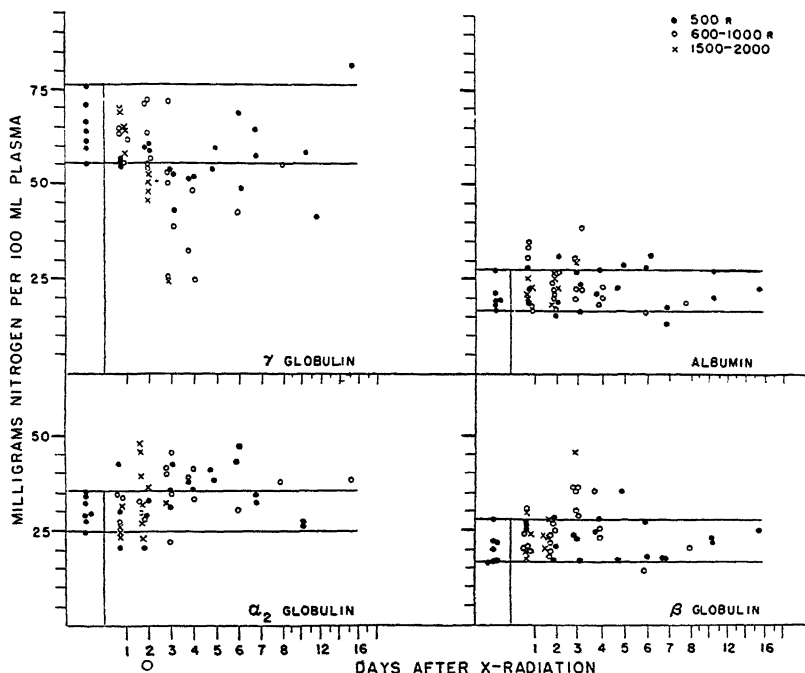


FIG. 3. The nitrogen contents of the protein components of Fraction II + III.

cedures of Sperry and Brand (4). Aliquots of the alcohol-acetone extract were evaporated to dryness, the residue was extracted with petroleum ether and the total lipid carbon was determined by the manometric technique of Van Slyke and Folch (5). Total nitrogen was determined by the micro-Kjeldahl procedure.

RESULTS

The nitrogen distribution of the four plasma fractions (I, II + III, IV-4, and V) of the control and of the X-radiated rats is shown graphically in Fig. 1. Practically no changes in the nitrogen content are observed in Fractions I and II + III. A marked increase of the nitrogen

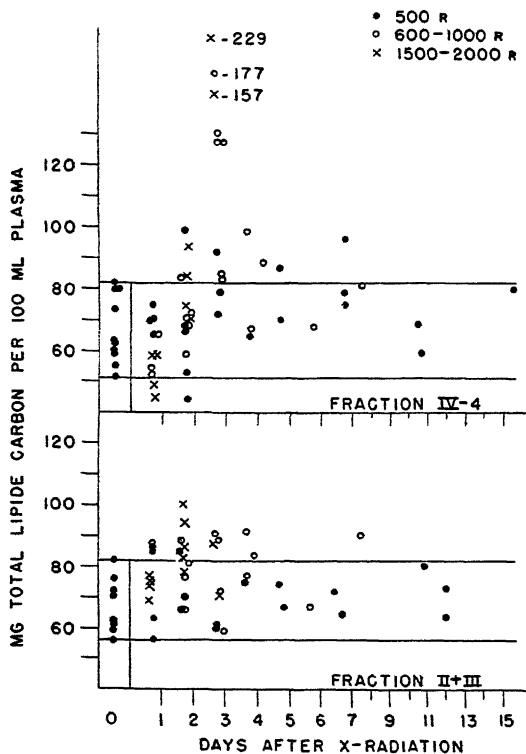


FIG. 4. The lipid-carbon contents of Fractions II + III and IV-4.

contents in Fraction IV-4 is seen after the second day, particularly at the higher dosages. None of the animals exposed to dosages of 1000 r or more survived more than 3 days. The decreases in the values of Fraction V are marked after moderate and large dosages of X-rays. In general, the changes in the protein contents of the four fractions are not exaggerated after large lethal doses of X-ray.

Electrophoresis

The patterns for Fraction IV-4 and V in control and experimental animals obtained by the modified fractionation procedure are practically identical with those shown previously (2). The percentage distribution of the components of Fractions IV-4 and V show no outstanding change after irradiation. This is surprising in view of the marked in-

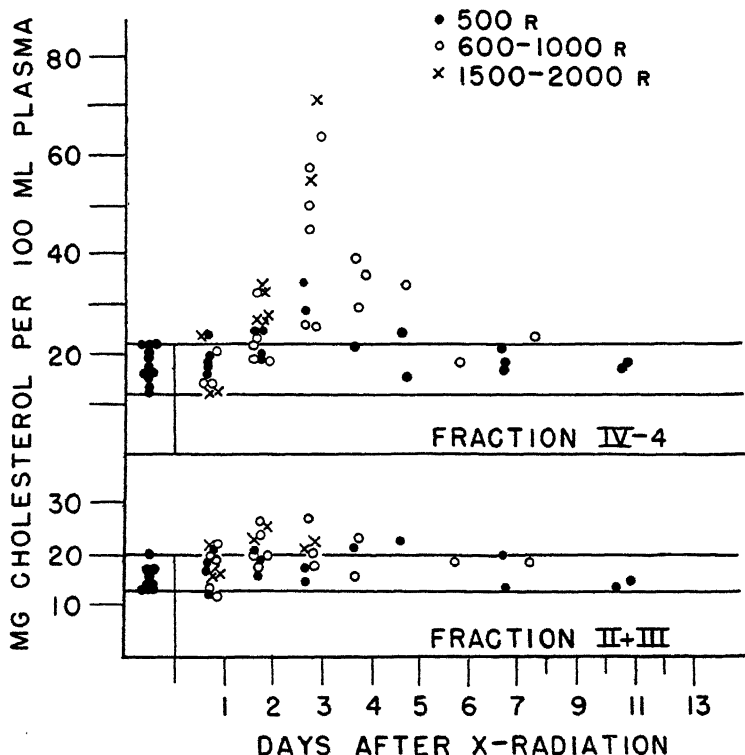


Fig. 5. Total cholesterol content of Fractions II + III and IV-4.

creases and decreases in nitrogen content of Fractions IV-4 and V, respectively.

Typical patterns of the ascending boundaries of Fraction II + III of the plasma of control and irradiated rats are shown in Fig. 2. The α_2 -globulin component is present in larger amounts than previously ob-

served (2). The components designated as α_1 and α_2 are poorly resolved in many patterns and are so designated as a matter of convenience. The outstanding change noted in this fraction after irradiation is the pronounced decrease in the γ -globulin component which is readily observed by inspection.

The nitrogen contents of each component in Fraction II + III are calculated from the total nitrogen and the percentage distribution of the respective components and the results are shown graphically in Fig. 3. The greatest changes in the γ -globulin content are observed in the animals exposed to doses greater than 500 r and are consistently lower on the 3rd and 4th postradiation days. It is interesting to observe that the values are within the control range on the first day regardless of the dose. Appreciable and consistent increases in the α_2 -globulin content are also seen after the 3rd day. Changes in the β -globulin and albumin contents are not striking.

Lipide Analyses

The data showing the effect of irradiation on the percentage concentrations of lipid carbon in Fractions II + III and IV-4 are shown in Fig. 4. Relatively small increases above the control range are observed on the 2nd, 3rd, and 4th days in Fraction II + III. Marked increases in the lipid carbon are seen in Fraction IV-4 during the terminal stages on the 3rd day following lethal X-ray exposures. The remaining values above the control range are only moderately increased. The total cholesterol concentrations are increased on the 2nd, 3rd, and 4th days in Fractions II + III and IV-4 (Fig. 5). The largest increases are observed in Fraction IV-4 on the 3rd postradiation day.

DISCUSSION

Of the four fractions studied, changes in the distribution of the protein components of the electrophoretic patterns are observed only in Fraction II + III. As a general rule, the variations from the control are minimal after exposure to a 500-r dose. After lethal doses of X-rays, a distinctive "injury" pattern is obtained in Fraction II + III which is similar to that previously described after thermal injury, and after the subcutaneous injection of turpentine and adrenal cortical hormones (2). These changes are not associated with any appreciable increases in the lipid content of this fraction. In previous work with rats injured by

heat and by injection of nitrogen mustard (3), the greatest changes in the lipid content are observed in Fraction II + III.

A latent period of at least 1 day is encountered before deviations from the respective control ranges are seen. Since involution of the spleen and thymus is pronounced 24 hr. after irradiation with nonlethal doses of X-rays, the changes noted in nitrogen and lipid contents and the variations in the electrophoretic pattern of Fraction II + III are probably not associated with the immediate effects of lymphoid tissue involution.

SUMMARY

The nitrogen and lipid contents of four plasma fractions are presented for rats exposed to nonlethal and lethal doses of X-rays. Pronounced increases in the nitrogen contents of the α_1 -globulin-rich Fraction IV-4 and decreases in the albumin-rich Fraction V are seen. The most pronounced changes in the total lipid and cholesterol content are noted in Fraction IV-4.

The percentage distribution of the components of the electrophoretic patterns of the four fractions do not deviate significantly from the controls except in the case of Fraction II + III. The γ -globulin content of this fraction is consistently diminished.

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Plastic House for the Quantitative Separation of Urine and Feces Excreted by Male Rats¹

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The various metabolism units designed for the separation of urine and feces in experiments with rats and other small animals have been reviewed briefly by Harned, Cunningham, and Gill (1). In our experience none of these units is entirely successful in preventing cross-contamination. Thus, none is satisfactory for excretion studies with radioactive isotopes, where contamination of urine with minute amounts of feces may introduce serious errors. The unit described below avoids this source of error since the feces are collected directly in a plastic container at the anus and the urine is funneled into a tube fixed below and around the penis.

Construction details are as shown in Fig. 1. When the feces collector (C) is in place, the shelf at the front supports the testicles of the rat and serves to prevent lateral displacement of the box. The collector is positioned by an auxilliary support rod (D), which is first attached to the back of the rat; two pieces of adhesive tape (encircling the abdomen and the base of the tail between the screws on the support rod) are sufficient. The tail passes through the feces collector. By passing the slot in this collector between the two washers on the support rod, and adjusting the nuts to give a close fit, the feces collector is fastened in place. The back end of the collector is closed with plastic tape.

The urine collector (B) is an 18-mm. test tube shortened to 4.5 in. and lipped. It is held in position by a $\frac{1}{8}$ -in. plastic sleeve, the side arm of which is fastened to the base of the feces collector using plastic tape. Spacer shims raise the lip of the tube to ensure a snug fit against the body.

¹ This investigation was aided by a grant from the Quaker Oats Company, Chicago, Illinois.

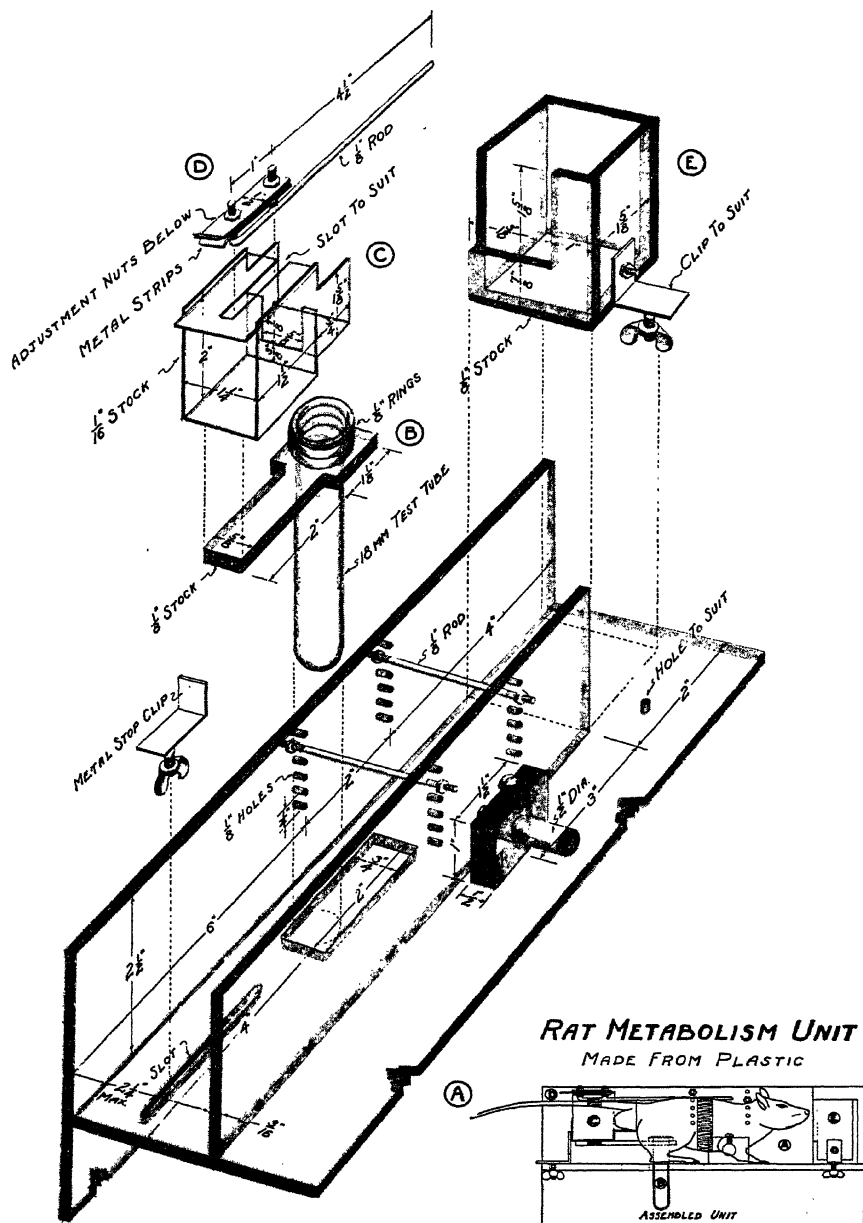


FIG. 1. Unit of plastic house for the collection of all urine and feces without cross-contamination.

To prevent the displacement of these collectors, the animal is housed in a plastic box (A), which is similar in principal to the tubular anti-coprophyagy cage designed by Geyer *et al.* (2). The width, depth, and height can be adjusted to the size of rats weighing more than 100 g. The rat is placed in the house so that the urine collector fits through a slot in the floor; the side is brought into position and secured by a screw; the rear clip is moved forward until it holds the feces collector in position; then rods are inserted at an appropriate level above the animal. When the food box (E) is in place the rat is effectively immobilized in an all-plastic compartment. Water is provided from an inverted bottle fitted with a glass nipple.

TABLE I

Distribution of Radioactive Calcium in the Urine and Feces Following the Ingestion of 1.49 mg. Ca (1.92×10^4 counts/min.) as the Lactate

| Time after ingestion | Per cent of dose excreted | | | | | |
|-------------------------|---------------------------|-------|-----------|-------|-----------|-------|
| | Rat no. 1 | | Rat no. 2 | | Rat no. 3 | |
| | Urine | Feces | Urine | Feces | Urine | Feces |
| hr. | | | | | | |
| 24 | 0.51 | 28.1 | 0.16 | 17.1 | 0.27 | 24.5 |
| 48 | nil | 0.5 | nil | 1.5 | nil | 0.9 |

Batteries of these units, constructed on a common base, conserve space and materials and are easier to manipulate. Cage units are fastened together using 1-in. corner irons; the parts of the food and feces collectors are joined with plastic cement. Rats have been kept in these cages for as long as 4 days. During the first 2 days they behaved normally and consumed the diet; later they refused to eat, and lost weight.

These units have been used in studies of the excretion of radioactive calcium (Ca^{45}) by rats following the ingestion of 1.49 mg. of calcium as the lactate.

The results of one experiment (Table I) demonstrate why complete separation of the excreta is important. Since approximately 100 times more Ca^{45} is excreted in the feces than in the urine, the slightest contamination of the urine by fecal material may cause considerable error.

SUMMARY

A plastic house suitable for the quantitative separation of urine and feces excreted by male rats is described. Collections during a period as long as 4 days have been successful. This apparatus is especially useful in studies of the absorption and excretion of radioactive compounds.

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Effect of Diet on the Reaction of Rabbit Erythrocytes to Nitrite *in vitro*

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INTRODUCTION

Methemoglobin (MHB) formed by addition of nitrite to erythrocytes *in vitro* is reduced in a few hours through the action of red cell substrates and respiratory enzymes (1-4). The rate of disappearance of MHB following a standard dose of sodium nitrite (NaNO_2) was used in recent studies (5) as a criterion of the metabolic activity of erythrocytes, and it was shown that cells obtained from weanling rabbits lose MHB more quickly than do erythrocytes from the adult host. An opportunity occurred to apply this procedure in studying corpuscles from rabbits fed purified diets (6). Herbivorous species in general are said to be resistant to methemoglobinemia (7) and so it was considered of interest to study the effect of alteration in diet on the rate of MHB formation or reduction after nitrite.

EXPERIMENTAL

Procedure

An inbred strain of albino New Zealand rabbits was used. Weanling male litter mates weighing between 1600 and 1800 g. were placed in individual metal cages. The test animals were fed a purified ration designated No. 672, unless stated otherwise, as described previously (6), and the controls received the ration fed the breeding colony.¹ Diet 672, although able to maintain many of the rabbits for a period of

¹ The purified ration contained the following substances expressed in g.-%: sucrose, 55.4; leached, alcohol-extracted casein, 20; cellophane, 15.6; Wesson oil, 5; and O & M salt mixture, 4. The supplements are expressed in mg.-%: choline, 200; carotin (7500 units/g.), 87; Drisdol (D_2 40,000 units/g.), 20; niacin, 20; inositol, 10; α -tocopherol, 7.5; calcium pantothenate, 1.5; thiamine, 0.7; riboflavine, 0.7; pyridoxine,

years, is not adequate for maximal growth, reproduction, and longevity. Erythrocytes were drawn by cardiac puncture into a few drops of purified heparin solution. Animals were not bled oftener than at monthly intervals. The cells were packed by 30 min. centrifugation at $1700\times g$, or were washed five times and packed and were then suspended in an equal volume of plasma, saline, or substrate medium as described in the earlier report (5). After addition of a standard amount of NaNO_2

TABLE I

Effect of Diet on the Disappearance of MHb from Nitrite-Treated Erythrocytes

| | Medium in which cells were suspended | Regimen | Time for MHb concentration to return to 3.0 g.-% | LSMD ^a ($p = 0.01$) | Max. rate of MHb reduction between peak and 3.0 g.-% | LSMD ^a ($p = 0.01$) |
|-----------------------|---|--------------------------------|--|----------------------------------|--|----------------------------------|
| Unwashed erythrocytes | Plasma plus glucose at 125 mg. % | Stock diet | hr. 3.30 | hr. 0.18 | g.-%/hr. 4.09 | g.-%/hr. 0.64 |
| | | Purified diet | 2.06 | | 5.29 | |
| | | Stock diet minus purified diet | 1.24 | | -1.20 | |
| | | | | | | |
| Washed erythrocytes | 0.85% NaCl plus glucose at 125 mg. % | Stock diet | 4.65 | 0.39 | 3.16 | 0.53 |
| | | Purified diet | 2.70 | | 4.12 | |
| | | Stock diet minus purified diet | 1.95 | | -0.96 | |
| | | | | | | |
| | 0.25 M Sodium lactate | Stock diet | 2.96 | 0.37 | 4.20 | 0.65 |
| | | Purified diet | 1.90 | | 6.38 | |
| | | Stock diet minus purified diet | 1.06 | | -2.18 | |
| | | | | | | |
| | Saline phosphate buffer plus glucose at 375 mg.-% | Stock diet | 4.30 | 0.47 | 3.09 | 0.43 |
| | | Purified diet | 3.13 | | 3.75 | |
| | | Stock diet minus purified diet | 1.17 | | -0.66 | |
| | | | | | | |

^a Least significant mean difference.

Flask contents: 2.0 ml. packed erythrocytes + 2.0 ml. suspending medium + 0.3 ml. 1% NaNO_2 . Mean values for rabbits on purified diet were derived from: 104 runs involving 44 animals for plasma, 125 runs with 54 animals for saline, 76 runs with 42 animals for lactate, and 74 runs with 46 animals for glucose. Mean values for rabbits on stock diet were derived from: 29 runs involving 24 animals for plasma, 44 runs with 35 animals for saline, 1 run on each of 42 animals for lactate and glucose.

0.7; 2-methyl-1,4-naphthoquinone, 0.075. The stock ration contained alfalfa leaf meal, 40%; pulverized No. 1 whole wheat, 30.5%; oat groats, 15%; soybean meal, 13%; and supplements of calcium carbonate, iodized sodium chloride, irradiated yeast, and cold pressed wheat germ oil, which was pressed into BB size pellets.

the reduction of MHb was followed in these cells, shaken gently at 38° in a 25-ml. paraffined flask. The procedure for determining MHb and computing the maximum rate of MHb reduction was that used previously (5). The term "MHb cells" refers to erythrocytes incubated 20 min. at 38° with about 0.15 ml. 1% NaNO₂/ml. of blood and then washed and packed in the routine manner.

Reversion of MHb after Addition of Nitrite

Results obtained on fresh unwashed blood samples as well as on washed erythrocytes from a number of rabbits 6 months of age or older fed the purified diet and a group of comparable age fed the stock diet are shown in Table I. Except for ten of the experimental and twelve of the control rabbits which were used in the studies on whole blood, different individuals were employed in the tests on washed corpuscles. It is apparent that MHb disappears more quickly from cells of the rabbits fed the purified diet, whether unwashed or washed corpuscles are used. In general, MHb persists about 50% longer in all animals fed the stock ration. The mean value of 1.90 hr. for the experimental diet animals with lactate agrees well with that of 1.88 hr. found previously for weanling animals with lactate (5).

Conversion from Purified to Stock Diet

A group of fourteen rabbits fed the experimental diet about 6 months and then transferred to the stock diet became slower in respect to rate of MHb disappearance when tested 8 months after change of diet. Thus the average time in hours for the MHb concentration to return to 3.0 g.-% was 1.8 before, and 3.7 after conversion.

Modification of Basic Diet

A number of 10-week-old rabbits on diet 672 were divided into four groups in an experiment to determine whether the results with purified diet might be due to inadequate intake of an essential dietary factor. One group served as purified diet controls, a second received 1% liver paste (Valentine's 1:20 concentrate) in the diet, a third received dried debittered brewers' yeast (Vita Food) at 5% for 3 months, and then 3% for 3 months, and the fourth received biotin at 8 µg.-% and folic acid at 0.4 mg.-%.

The rate of MHb disappearance with both lactate and glucose as substrate was determined before starting the diet, and 6, 9, and 12

months after. Although the results obtained were nearly comparable with either substrate, only the data with lactate are presented (Table II). In contrast to the stock diet controls of the same age, none of the experimental animals changed after 6 months on the described regime. Kale, then fed *ad lib.*, to the first three animals in each group was

TABLE II

Effect of Supplementing Basic Diet on Time, in Hours, for MHb to Disappear

Flasks contain 2.0 ml. packed washed cells of one rabbit + 2.0 ml. 0.25 *M* sodium lactate + 0.3 ml. 1% NaNO₂.

| | Age in months | | | | Age in months | | | |
|--|---------------|------|------|------|------------------|------|------|------|
| | 2.5 | 9 | 12 | 15 | 2.5 | 9 | 12 | 15 |
| Liver supplement | | | | | Yeast supplement | | | |
| No. of animals | 8 | 8 | 5 | 5 | 7 | 7 | 5 | 5 |
| Avg. time for [MHb] to return to 3.0 g.-% | 2.05 | 2.03 | 2.28 | 2.17 | 1.87 | 1.76 | 1.78 | 1.95 |
| Avg. body wt., <i>kg.</i> | 1.75 | 2.59 | 3.30 | 3.39 | 1.70 | 2.42 | 3.05 | 3.05 |
| Biotin and folic acid | | | | | No supplement | | | |
| No. of animals | 8 | 8 | 7 | 6 | 9 | 9 | 7 | 7 |
| Avg. time for [MHb] to return to 3.0 g.-% | 1.77 | 1.97 | 1.69 | 1.85 | 1.91 | 1.83 | 1.61 | 1.76 |
| Avg. body wt., <i>kg.</i> | 1.46 | 2.64 | 2.78 | 2.91 | 1.54 | 2.68 | 2.93 | 2.97 |
| Stock diet controls | | | | | | | | |
| No. of animals | 12 | 12 | 11 | 11 | | | | |
| Avg. time for [MHb] to return to 3.0 g.-% | 1.85 | 3.05 | 2.82 | 2.85 | | | | |
| Avg. body wt., <i>kg.</i> | 1.76 | 3.85 | 4.21 | 4.29 | | | | |

likewise without effect after 3 months. At this point all supplements were withdrawn and ground whole wheat was made 30% of the ration of half the survivors in such a manner that the ratio of protein, carbohydrate, and fat was not disturbed. The whole wheat, following a 3-month trial, also appeared to be without effect.

Effect of Caloric Intake on MHb Reversion

Since the various qualitative dietary changes were without effect on MHb reversion, it was considered that the findings might be due to restricted caloric intake. The surviving animals in the previous experiment were placed on the stock diet, with half of them restricted to a caloric intake commensurate with that on the purified diet, *i.e.*, 60 g. of the stock ration per day, and the other half receiving all they could consume. It was found that the red cells of animals in the restricted group failed to change in 3 months on this regimen, whereas those of rabbits fed the stock diet *ad lib.* became somewhat more sluggish in losing MHb. Thus the average time required for the MHb concentration to return to 3.0 g.-% was 2.2 hr. both before and after conversion for the eight animals in the restricted group, whereas it was 1.86 and 2.41 hr., respectively, for the nine animals in the *ad lib.* group. Unlike the rabbits in the earlier experiment which had been on the purified diet 6 months before conversion to the stock ration, these maintained a year on the experimental diet did not change to the extent which has been found characteristic of normal aging.

MHb Formation

The time for MHb to disappear after nitrite may depend not only on the rate of MHb reduction but also on the rate of its formation. Accordingly an attempt was made to compare the various dietary groups of the preceding section with respect to speed of MHb formation. In this experiment equal amounts of the washed cells of two or occasionally three animals were combined in preparing the flask for each diet group, and the four groups were tested together at room temperature. The results of four such runs, which included all the available animals after 6 months on the diet, were averaged to obtain the curves for the experimental diets shown in Fig. 1. Comparable data were obtained in an experiment in which pooled blood of all the 7 to 9 animals in each group was tested. The curve for the rabbits on stock diet is that reported previously (5). The rate of MHb formation was measured also in rabbits converted to the stock diet. The first of the three curves on converted animals represents the average of four tests on pooled blood from 12 of the animals studied in the first experiment on conversion to stock diet. The second of this series of curves, obtained 6 months after conversion to stock diet, depicts MHb

formation in the pooled blood of the rabbits fed calories *ad lib.* in the second conversion experiment. The third curve was derived from a simultaneous test on the group restricted in calories. Methemoglobin formation occurs more rapidly and the concentration reached is greater in the intact cells of the purified diet groups than in the stock diet controls with the exception that the cells of liver-supplemented animals resemble those of the controls. It may be said that MHb appears more slowly and attains a lower concentration in animals converted to stock diet than in those maintained on this diet throughout. As compared with the differences for intact cells there is only a slight difference in the rate of MHb formation in hemolysates of the several groups.

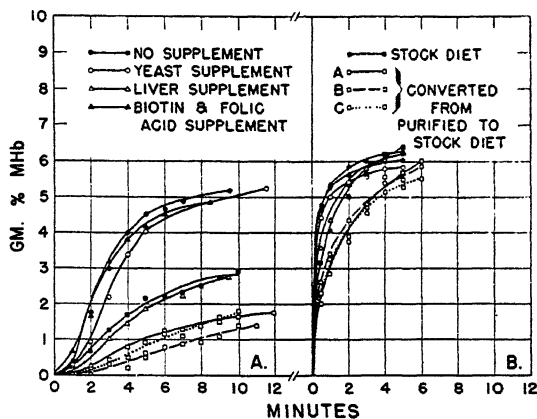


FIG. 1. Effect of diet on rate of MHb formation by nitrite in intact and hemolyzed washed erythrocytes. Flask contents: A, intact cells: 1.0 ml. cells + 3.0 ml. normal saline + 0.075 ml. 1% NaNO_2 ; B, hemolysates: 1.0 ml. cells + 3.0 ml. distilled water + 0.075 ml. 1% NaNO_2 .

However, the differences that do appear to exist are in the same direction in the hemolysates as in intact cells.

Reduction in MHb Cells

In order to measure MHb reduction unopposed by MHb formation, reduction in "MHb cells" was studied. As shown previously, the reduction of MHb in rabbit cells resembles a first-order reaction (5). Therefore, the average hourly decrease in the log of the MHb concentration for five to ten samples taken during the course of reduction

to 3.0 g.-% (K_1) was employed in comparing MHB reduction in cells of control and experimental diet animals. With lactate or fumarate as substrate a rate about one-third faster was obtained for the purified diet group. In runs with glucose no such difference was obtained. Anaerobic manometric measurements of the rate of lactic acid formation in MHB-free red cells were made simultaneously with many of these tests according to the method described previously (8). Approximately the same difference in rate of fumarate utilization was obtained by this method as with MHB reduction. The purified diet groups gave a somewhat slower rate with glucose as substrate in manometric runs when compared with a large number of adult control rabbits or with themselves at the time of starting on the experimental diet.

DISCUSSION

Since MHB reversion occurred rapidly in the purified diet groups despite addition of various crude supplements and in the stock diet group fed limited calories, it seems probable that the accelerated disappearance of MHB is related to the caloric restriction. The caloric consumption on the experimental ration amounted to approximately 60% of that on the stock diet, exclusive of the kale ingested. Weanling rabbits and those fed purified diet for many months are similar on the one hand in having red cells that lose MHB quickly after nitrite, and on the other in lacking any grossly-visible fat deposits at autopsy. Possibly deposition of lipide or polysaccharide material in erythrocytes as the rabbit matures on an adequate diet results in the slowing of metabolic processes. A relationship is suggested between this retardation of a normal maturing process induced by caloric restriction and the failure of rats to mature when fed insufficient calories for normal growth (9).

The rate of MHB formation appears to be determined on a different basis since the rate was retarded in the purified diet group fed liver supplement, and since on conversion to stock diet the rate was equally slow in the group fed *ad lib.* and the group with restricted calories. Here it would seem the purified diet is lacking in an unidentified factor present in the liver paste and stock ration. Whether the factor is one which effects cell permeability to nitrite, or one which incorporated as a nondialyzable constituent of the red corpuscle reacts with nitrite thus protecting the Hb, remains uncertain. The much faster rate of

Hb oxidation in hemolysates than in intact cells might result from the cell membrane acting as a barrier between Hb and nitrite. Since the difference between hemolysate and intact cell is decreased in corpuscles of animals receiving the purified diet, presumably the barrier to nitrite is less effective here. However, in hemolysates, Hb oxidation takes place slightly more rapidly in rabbits on purified diet and more slowly in converted rabbits than in those fed the stock ration or the purified diet plus liver. These differences can most reasonably be attributed to temporary adsorption of the nitrite by a substance other than Hb. The permeability and the adsorption concepts could be resolved into one thesis by suggesting that the red cell membrane itself adsorbs nitrite and does so least actively in purified diet and most actively in converted rabbits. The disrupted membrane in the red cell hemolysate, although still adsorbing nitrite to a different extent in the different dietary groups, would be less accessible to nitrite while the hemoglobin has become more accessible than in the intact cell.

It is noteworthy that the corpuscles of a herbivorous species, characteristically resistant to the methemoglobinogenic action of nitrite (10), have been rendered more susceptible to this effect by the feeding of an experimental diet.

ACKNOWLEDGMENTS

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SUMMARY

The effect of the type of diet consumed by the animal host on the rate of formation and disappearance of methemoglobin (MHb) after addition of a standard amount of nitrite to erythrocytes has been investigated.

1. The time required for MHb to disappear from whole blood or washed erythrocytes is less for rabbits fed a purified diet than for those maintained on a stock diet.

2. Liver paste, yeast powder, ground whole wheat, or kale incorporated in the purified ration failed to alter the rate of MHb reversion.

3. Erythrocytes of animals converted from the purified to the stock diet undergo slowing of MHb reversion when the rabbits are allowed adequate calories.

4. Methemoglobin reduction by lactate and fumarate proceeds at a greater rate in MHb cells of rabbits given the purified diet.
5. The rate of MHb formation is relatively fast in cells of rabbits receiving the purified diet.
6. Liver paste added to the experimental diet retards MHb formation to a rate equal that for rabbits on stock diet.
7. MHb formation occurs most slowly in cells of animals converted from the purified to the stock ration.

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Studies on the Microbiological Degradation of Wool.

II. Nitrogen Metabolism

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INTRODUCTION

A previous paper from this laboratory (1) has discussed the sulfur metabolism of the dermatophytic fungus *Microsporium gypseum* in the degradation of wool. The present report is concerned with the changes in the nitrogen of the wool in the same process.

EXPERIMENTAL

A. Materials and Analytical Methods

Scoured sheep wool, treated as described in (1) was used. The fungus used was *Microsporium gypseum*, QM-196, which had been isolated from deteriorating woolen fabric. The basic medium for the shake-flask-technique studies was a suspension of wool in salt solution, made up as follows: $MgCl_2 \cdot 6H_2O$, 1.68 g.; K_2HPO_4 , 2.09 g.; KH_2PO_4 , 2.68 g., and 50.0 g. wool, made up to 1-l. with distilled water. The wool served as the only source of carbon, nitrogen, and sulfur.

Nitrogen was determined by the micro-Kjeldahl method; amino nitrogen by the Van Slyke manometric procedure (2); amino nitrogen on the solid wool residue was determined by the Dougherty and Ogg modification (3) of the Van Slyke manometric method. Amide nitrogen was determined after gentle hydrolysis with 1 N H_2SO_4 for 3 hr. in a boiling water bath. Reagents for ammonia nitrogen and amide nitrogen were those as described by Pucher, Vickery, and Leavenworth (4).

Fractionation of the protein in the digest was carried out by the method of Was-teney and Borsook (5) which involves precipitation of the protein by trichloroacetic acid, of the proteases by sodium sulfate at 33°C., and of the peptones by tannic acid, all under definitely fixed conditions.

B. Shake-Flask Technique for Study of Action of Fungus on Wool

This technique was described in detail in a previous publication (1). It consisted essentially of a system whereby sterile washed air was blown through a large 22-l. flask containing 5 l. of the basic salts medium and 5% wool. The ammonia evolved

from the main reactor vessel was absorbed in saturated boric acid. Each time a sample of the metabolic mixture was withdrawn for analyses, the absorbers were replaced by fresh ones.

To supplement and improve the accuracy of the results obtained from the above experiment, a series of eighteen 1-l. Florence flasks containing 150 ml. nutrient salt solution and 7.5 g. of chopped wool were prepared, sterilized, and inoculated with *M. gypseum*. At 0, 3, 6, 10, and 14 days, 4 flasks were removed and the wool plus mycelium residues of each of 2 flasks were thoroughly washed, dried, and analyzed separately. The remaining two flasks were used for duplicate determination of mycelium by digestion with 10% NaOH as previously described in (1).

RESULTS

A. Gross Nitrogen Changes During Degradation of Wool in Shake Flasks

Data in Fig. 1 and 2 show that the increase in alkalinity in the shake-culture is coincident with an accumulation of soluble-N compounds in the filtrate. The highest pH is noted at the 11th day, and after that

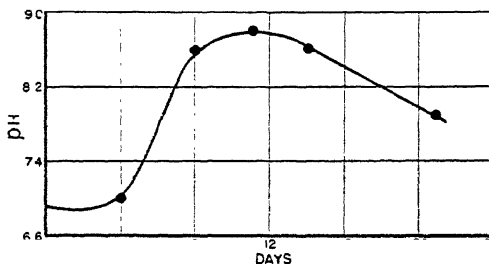


Fig. 1. pH changes in metabolic filtrate of *M. gypseum* growing on wool.

time the pH declines. As soon as the maximum pH is reached, active breakdown of the wool ceases as is indicated by no further loss of wool residue after the 14th day.

Figure 2 shows the trend of daily changes in the nitrogen of the wool after the growth of the fungus upon it. The curve relating to gases is plotted as nitrogen obtained by calculation from the ammonia evolved by the reaction, and is cumulative.

Subsequent studies on a series of filtrates have shown that about 50% of the total nitrogen of the filtrate was in the form of ammonia nitrogen. After the 14th day, the solubilization of wool was almost completely stopped.

Since the data in Fig. 2 do not account for all the nitrogen, a somewhat different experiment was set up to simplify and possibly reduce the errors that come from sampling one large vessel over a period of 21 days. A series of liter flasks containing 150 ml. salt solution plus 7.5 g. of chopped wool were set up and inoculated. The simple shake-flask technique replaced the constant air stream used in the former experiment. At intervals flasks were taken off and the contents of each ana-

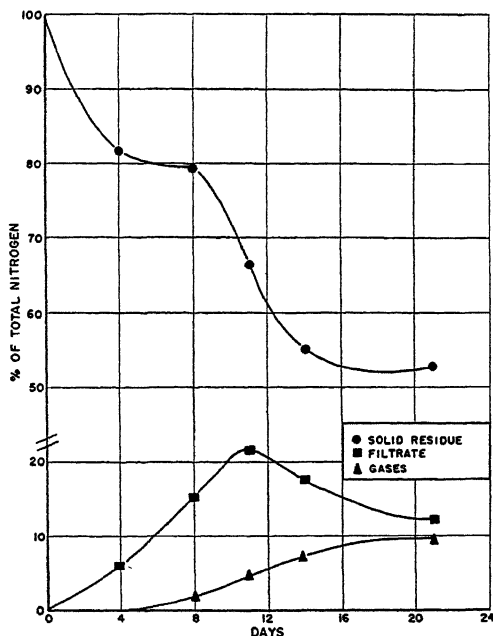


Fig. 2. Nitrogen distribution during wool breakdown by *M. gypsum*.

lyzed separately. The results appear in Table I. All of the values are calculated to an ash-free, moisture-free, basis.

In order to make the calculations in Table I on the basis of the *wool residue* rather than the *wool plus mycelium residue*, analyses of three batches of *M. gypsum* mycelium, grown on glucose-peptone-mineral salts medium for 10 days, were made. Average values, calculated to an ash-free, moisture-free, basis gave N, 6.28%; amino N, 0.1%; S, 0.327%; and cystine, 0.276%. It is assumed that the N and the S of

TABLE I

Analytical Values on Wool Residue at Various Stages in Digestion by M. gypseum

| Days | Weight of wool remaining | Weight of mycelium | Amino N | N | S | Cystine | Ratio | |
|------|--------------------------|--------------------|----------------|------------|-----------|-----------|-------|-----------|
| | | | | | | | N/S | S/Cystine |
| 0 | 7.090 | 0.000 | mg./g. 3.78 | % 16.50 | % 2.99 | % 8.55 | 5.51 | 0.350 |
| 3 | 6.499 | 0.201 | 3.60 | 16.53 | 3.10 | 9.26 | 5.33 | 0.335 |
| 6 | 5.829 | 0.541 | 3.29 | 16.38 | 3.34 | 9.95 | 4.90 | 0.335 |
| 10 | 4.956 | 0.644 | 3.08 | 16.68 | 3.66 | 10.45 | 4.56 | 0.350 |
| 14 | 4.654 | 0.546 | 2.91 | 16.38 | 3.48 | 10.29 | 4.70 | 0.338 |

the mycelium do not vary significantly during the phases of the growth cycle on wool. It is further assumed in the following calculations that these values for mycelium grown on glucose-peptone medium are of essentially the same order of magnitude as when the organism is grown on wool. However, it is recognized that the composition of the mycelium may vary within fairly wide limits and even with a variation by a factor of 2, the S and N values given in Table I would not vary significantly.

The assumptions used in reporting the values in Table I may be best indicated by a sample calculation:

10-Day Digestion Sample

Total residue (wool + mycelium) = 5.600 g.

Wt. of mycelium = 0.644 g.

Wool residue = 4.956 g.

Nitrogen calculations:

$5.600 \times 0.1549^1 = 0.8674$ g. N in total residue

$0.644 \times 0.0628 = 0.0404$ g. N in mycelium

0.8270 g. N in wool residue

$\frac{0.8270}{4.956} \times 100 = 16.68\%$ N in wool residue alone.

Sulfur and cystine calculations were made in a similar manner.

It also may be pointed out that a linear relationship exists between the percentage of S and the log of time, and the N percentage does

¹ Total N/g. wool residue plus mycelium.

not change. The correlation coefficient between the former equalled 0.85. Thus there is a significant increase in the S percentage of the wool residue with time, but this is not true for the N values.

Since the amino-N values are of certain usefulness in our interpretation, they are given in Table II, and were values obtained on the same samples used to obtain the data in Table I. They were similarly corrected as above to give values for the wool residue, and not wool plus mycelium residue. Since only negligible amounts of amino N could be detected in mycelium, it was assumed that all was present in the wool and calculated as such.

Additional data showing an increase in the sulfur and cystine in the wool residue during digestion is shown in Table III, which compares

TABLE II

Changes in Amino-Nitrogen Content of Wool During Digestion by M. gypseum

| Days | Amino N | | |
|------|-------------------------|-------------------------------------|----------------------|
| | Mgs./g. of wool residue | Per cent of total N in wool residue | Change |
| 0 | 3.78 | 2.29 | <i>per cent</i> — |
| 3 | 3.60 | 2.26 | — 1.3 |
| 6 | 3.29 | 2.24 | — 2.2 |
| 10 | 3.08 | 2.15 | — 6.1 |
| 14 | 2.91 | 2.03 | —11.4 |

the action of a cell-free metabolic filtrate having high activity on *ball-milled* wool, as well as a commercial trypsin and a crystalline trypsin sample. Other experiments have confirmed the fact that the difference in degree of digestion between the fungal enzyme extract and crystalline trypsin is due to the presence of additional proteinases in the former. It is suggested that this is the same type of digestion as goes on with the more intact chopped wool.

The initial amino-N value of 3.78 mg./g. (Table I) is high in comparison with that of "normal" wool. This wool differed from the "normal" wool since it had been autoclaved, as described in the *Methods* section. Since a consistent value of 2.96 mg./g. for nonautoclaved wool

TABLE III

Analysis of Residues From Digestion of Ball-Milled Wool With Enzyme Extracts of M. gypseum or by Trypsin ^a

| Enzyme | Digestion of ball-milled wool | Cystine, % | | Sulfur, % | | S/Cystine ratio |
|---------------------------------------|-------------------------------|------------|-------------------|-----------|-------------------|-----------------|
| | | Residue | Lost on digestion | Residue | Lost on digestion | |
| None | — | 8.17 | % | 2.78 | % | 0.34 |
| Enzyme extract from <i>M. gypseum</i> | 83.0 | 9.28 | 79.7 | 4.7 | 69.8 | 0.50 |
| Enzyme extract from <i>M. gypseum</i> | 88.1 | 12.67 | 81.6 | 3.8 | 83.8 | 0.30 |
| Crude trypsin, 0.05% | 76.1 | 11.96 | 65.0 | — | — | — |
| Crystalline trypsin, 0.01% | 61.5 | 12.83 | 40.0 | 4.45 | 38.5 | 0.35 |
| Crystalline trypsin, 0.05% | 60.5 | — | — | — | — | — |

^a After 4 days incubation.

was found, it was of interest to compare our normal value with other normal values for wool as found in the literature. They are as follows:

| Wool | Mg./g. | Reference |
|---------|--------|-----------|
| Intact | 3.05 | (6) |
| Chopped | 3.30 | (3) |
| Chopped | 3.16 | (7) |

B. Nitrogen Partition of the Metabolic Filtrate

Results of a more detailed gross-N partition of the metabolic filtrate are summarized in Table IV.

Since it was known that a certain portion of the chopped wool was soluble in the salt solution alone, a control nitrogen partition was determined. However, this sample contained only 2% of the nitrogen of the inoculated sample.

Table IV shows that an amino nitrogen value of 0.44 mg. N/ml. was found. The total protein, proteose, peptone, and residual nitrogen was 0.97 mg. N, of which 71% or 0.69 mg. was the residual nitrogen fraction, which would be the nitrogen of the amino acids and dialyzable peptides. The amino nitrogen would then represent 45.4% of this total nitrogen. This figure may be considered reasonable in view of the large portion of the amino acids such as arginine, histidine, lysine, and proline which make up 23% of the amino acid content of wool and contain other nitrogen than amino nitrogen. Furthermore, a portion of the nitrogen of this fraction is linked as peptide nitrogen.

TABLE IV
Fractional Analysis of Metabolic Filtrate

| | Uninoculated control | Fungal digest | | |
|--|----------------------|---------------|---------------------|-----------------|
| | | | Per cent of total N | Per cent of (A) |
| | mg. N/ml. | mg. N/ml. | | |
| Total N | 0.0563 | 2.68 | — | |
| Ammonia N | 0.003 | 1.62 | 60.4 | |
| Amide N | 0.000 | 0.09 | 3.4 | |
| Amino N | — | 0.44 | 16.4 | 45.4 |
| Protein, proteose, peptone, and amino acid N (A) | 0.0560 | 0.97 | | 100 |
| Protein N | 0.0040 | 0.04 | | 4.1 |
| Proteose N | 0.0004 | 0.18 | | 18.6 |
| Peptone N | 0.0072 | 0.06 | | 6.2 |
| Residual N (amino acid N and peptide N) | 0.0444 | 0.69 | | 71.1 |

The fact that wool is not so strongly acid despite its large amounts of aspartic and glutamic acid indicate the presence of acid amides. The usual amino acid analyses of wool seldom include analyses for either amide-N or glutamine or asparagine. Thus, the presence of a small (3.4% of the total), but definite amount of amide-N in the fungal filtrate indicates release of either asparagine or glutamine as such from the wool or a synthesis of this linkage by the action of the microorganism. This value should be considered a minimum figure since the wool was autoclaved for 5 min. at 120°C. to obtain a sterile substrate for the

action of the fungus and it has been shown by Hamilton (8) that glutamine at pH 6.5 is converted to pyrrolidonecarboxylic acid during autoclaving. This N would not be released by gentle acid hydrolysis, and as such would not be detected in an amide-N determination.

DISCUSSION

The following observations regarding wool residue at various stages of digestion by *M. gypseum* can be made from the data in Table I:

1. The nitrogen content does not show a significant variation with time.

2. The sulfur content shows a significant constant increase with time of digestion.

3. The S/cystine ratio stays fairly constant. This fact indicates that the sulfur is still linked in the cystine of the wool as before any microbial action. The above results may be interpreted as meaning that since the sulfur and cystine apparently increased, this increase was brought about by a differential hydrolysis of the wool protein. Thus a nitrogenous component with less cystine per original unit weight of wool is split off first. This is reflected in Table I as keeping the nitrogen constant and the sulfur increasing. A further example to illustrate this point may be given by taking the 14-day nitrogen value in wool residue from Fig. 1 and comparing it with the 14-day sulfur value for wool residue, given in a previous publication (1), and part of the same general experiment. The amount of the total nitrogen remaining in the wool residue was 55% as compared with 75% of the original sulfur.

4. It appears that deamination takes place primarily at the expense of the polypeptide after hydrolysis of the keratin molecule. Direct deamination of the intact keratin molecule accounts for little, if any, of the ammonia liberated. These statements are based on the analyses which show only 0.25% loss of amino nitrogen in the wool residue whereas there is 9.6% of the total nitrogen found as ammonia nitrogen in the filtrate after 21 days.

It was also found that a significant increase in the initial amino nitrogen of wool occurred as a result of autoclaving wool. This is probably due to a splitting of the polypeptide chains. This increase may have some significance in explaining the much greater susceptibility of autoclaved wool to microbiological degradation (9). More specifically,

it may suggest that the rate-governing step in the sequence of reactions involved in the degradation of wool is the initial cleavage of the peptide bond in the keratin molecule.

Mention might be made of the presence of a nondialyzable brown pigment always found present in the filtrate. In the Tiselius electrophoresis apparatus, the pigment and other small amounts of protein migrate faster than a main protein component. We have found this main protein fraction to have definite proteolytic activity against such substrates as ball-milled wool, gelatin, and casein.

SUMMARY

1. Degradation of wool by the action of the fungus *M. gypseum*, observed by means of shake-culture technique, was followed by means of nitrogen distribution studies.

2. At about 11 days, growth had stopped, and the quantities of soluble organic nitrogen and ammonia became fairly constant. The maximum digestion of the wool was reached in 14 days while the maximum liberation of soluble organic nitrogen into the filtrate occurred in 11 days.

3. The total nitrogen in the wool residue at various stages of digestion by *M. gypseum* does not significantly vary from its initial value. However, the sulfur values showed a constant increase with time of digestion over a period of 14 days, indicating a differential hydrolysis of wool protein.

4. A nitrogen partition of the metabolic filtrate showed that the 36% organic nitrogen was distributed as follows: 4.1% soluble-protein-N, 18.6% proteose-N, 6.2% peptone-N, and 71.1% amino acid- and peptide-N. The inorganic-N comprising 60.4% of the total N was ammonia.

5. Wool is not significantly deaminated until degradation of the molecule has proceeded to the stage of polypeptides and amino acids.

6. In the microbiological degradation of the wool molecule, there is a differential initial splitting away of non-sulfur portions.

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The Discovery of Sulfur in Penicillin

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In the chemical investigation of natural products, the identification of elements is of paramount importance. In this connection it is interesting to recall how often the presence of sulfur has been overlooked in important representatives of this class. This was for instance the case in the earlier stages of the isolation studies on penicillin, and with "sympectothione" (ergothionine) and thiamine even after their isolation in substantially pure form. Whatever were the causes for the failure to detect this element in the cases mentioned, it is pertinent to point out that the elementary analysis of the pure product and of its derivatives is not likely to reveal the error; thus, because of the equivalence in atomic weight of two oxygens to one sulfur, rational and correct figures are nevertheless obtained in the calculation of the atomic ratios of the other elements present. Such was actually the case with certain derivatives of penicillin [penicillamine, (2) penillic acid (3), penillamine (4)] before penicillin itself, then believed to be free of sulfur (1, 2), had been isolated in the pure state. The following account of how the presence of sulfur in penicillin was demonstrated by us [in July, 1943, the month in which this discovery was made independently also by the Oxford workers (4)] might be of interest to workers in the field.

In the earlier stages of the penicillin studies carried out in the Squibb Institute during the war, no attempts were made to characterize analytically the relatively crude preparations then at hand. The presence of sulfur was occasionally noted, but ascribed no significance in view of the prior publications reporting the absence of this element. Later, by means of a purification method employing acetone, a nearly colorless, well-particulated, but still amorphous sodium salt (5) was obtained, the properties of which suggested that its analysis might yield significant information. In order to save material which was very precious

at that time (July 19, 1943), the carbon and hydrogen analysis was combined with the sodium determination, *i.e.*, the weighed residue of the combustion sample, presumed to consist of a mixture of sodium oxide and sodium carbonate, was converted into sodium sulfate by treatment with sulfuric acid in order to ascertain the amount of carbon dioxide retained in the ash and thus to arrive at the total carbon dioxide formed in the combustion. The experimental figures were as follows:

| | |
|--------------------------|-------|
| Sample wt. | 4.867 |
| Wt. of untreated residue | 0.993 |
| Wt. of sulfated residue | 0.997 |
| Wt. H ₂ O | 2.17 |
| Wt. CO ₂ | 9.34 |

Since there was no significant weight change on sulfating the ash from the combustion analysis, it appeared that it had been already converted to sodium sulfate during this operation, and hence that the penicillin salt contained sulfur. This was confirmed by the determination of this element, which showed that sulfur was present in the proportion 1 sulfur:2 nitrogen:4 oxygen:1 sodium. Shortly thereafter the sodium salt was obtained in crystalline form, and its empirical formula $C_{16}H_{17}O_4-N_2SNa$ (sodium benzylpenicillinate) established.

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The Determination of Triose Phosphate Isomerase ¹

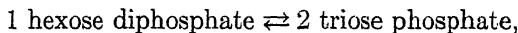
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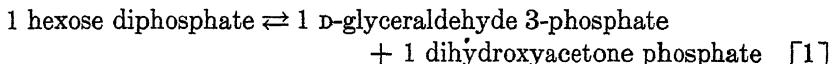
Received April 4, 1950

INTRODUCTION

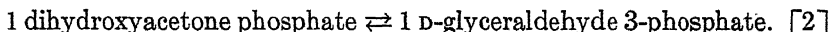
The zymohexase reaction,



which was first described by O. Meyerhof and K. Lohmann (1), was later on shown to consist of two partial reactions (2): The aldolase reaction:



and the isomerase reaction:



Since that time both enzymes have been purified and the aldolase from muscle has been crystallized by various authors. In assaying for the content of zymohexase in cells and tissues as well as in blood, all authors rely on the determination of aldolase. This seems justified in so far as Meyerhof and Beck (3) found isomerase in muscle in a very large excess over aldolase. However, because the isomerase content has not been determined in other tissues and cells and plays an equally important part in carbohydrate metabolism, we investigated anew the activity of this enzyme. This activity has so far been determined according to Meyerhof and Kiessling (4) or Warburg and Christian (7). Both procedures measure the speed with which glyceraldehyde phos-

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phate is transformed into dihydroxyacetone phosphate. Since the reverse reaction is the important one in the normal course of fermentation and glycolysis, it seemed worthwhile to develop a method for measuring the rate of the reaction in the opposite direction, and to find out whether the rates of reaction in both directions were consistent with the observed equilibrium constant (5). When this information had been obtained, the isomerase content of a number of different tissues was determined.

METHODS

The rate of transformation of glyceraldehyde phosphate into dihydroxyacetone phosphate was measured by determining alkali-labile phosphorus before and after treatment with weakly-alkaline iodine [see (3) and (4)]. It should be noted that some of our preparations of triose phosphate, particularly those which have been stored for several months, contained an impurity which analyzes as glyceraldehyde phosphate by the iodine method, but which is not converted to dihydroxyacetone phosphate by isomerase. This impurity is removed by reprecipitation of the barium salt between 27–40% alcohol [cf. (6)]; its presence is recognized by the fact that the isomerase equilibrium appears to be reached with more than 5% glyceraldehyde phosphate present.

In a few cases, conversion of glyceraldehyde phosphate to dihydroxyacetone phosphate was also followed spectrophotometrically, according to Warburg's first procedure (7).

The rate of the reverse reaction, from dihydroxyacetone phosphate to glyceraldehyde phosphate, was determined with a Beckman spectrophotometer. Each cuvette contained approximately 3.5 ml. of solution, buffered with veronal-acetate (usually at pH 7–7.5). The solution also contained 0.4 ml. of 5.4% Na_2HAsO_4 , approximately 1.5 μ mole of diphosphopyridine nucleotide (DPN), and a large excess of Warburg's "oxidizing enzyme," triose phosphate dehydrogenase from yeast. To this mixture were added the dihydroxyacetone phosphate, and, last of all, the isomerase. The glyceraldehyde phosphate formed is immediately oxidized, and the accompanying reduction of the DPN is followed by the development of the absorption band at 340 μ .

MATERIALS AND PREPARATIONS

Triose phosphate with the content of 60–80% D-glyceraldehyde 3-phosphate was prepared according to Meyerhof (6) [see also (5)]. Triose phosphate isomerase, and Warburg's oxidizing enzyme (from yeast) were prepared by the usual methods (3,5,8); the DPN was purchased from Schwarz laboratories (47% pure) and somewhat purified by precipitation with alcohol and acetone (final purity, 60%).

Dihydroxyacetone phosphate had previously been prepared synthetically from dihydroxyacetone (9), but a new and simpler enzymatic

method was employed to produce the samples used here: 1 g. barium triose phosphate (7.0% alkali-labile P, of which 65% was glyceraldehyde phosphate) was dissolved, the barium precipitated, and the neutral solution brought to 60 ml. To this, 20 ml. of veronal-acetate buffer (pH approx. 7.5) and 2 ml. of isomerase (sufficient to turn over 300 mg. P/min.) were added, and the mixture incubated 10 min. at 25°. The reaction was stopped with 7 ml. 40% trichloroacetic acid (TCA), and the protein precipitate centrifuged out. The supernatant was analyzed for glyceraldehyde phosphate, both in order to be sure that the isomerase reaction had gone to completion, and to find the total glyceraldehyde phosphate remaining. A 20% excess of iodine (in this case 2 ml. 1 *N*) was added to oxidize the glyceraldehyde phosphate in slightly alkaline solution (as in the determination of glyceraldehyde phosphate); after the oxidation had been completed, the solution was acidified, the iodine discharged with bisulfite, and the sulfate precipitated with barium acetate. The solution was neutralized, and the barium phosphate centrifuged off. Two fractions of dihydroxyacetone phosphate were collected, the first one by adding 27% alcohol and 1% ether (by volume) and the second one by bringing the alcohol concentration to 40%, and adding 100% acetone. The analyses on one preparation were as follows:

| | Fraction I | Fraction II |
|-------------------------|--------------|--------------|
| Total organic P | 5.18% | 7.56% |
| Alkali-labile organic P | 2.90 (= 56%) | 6.44 (= 85%) |
| Inorganic P | 3.57 | 1.21 |

The total yield (based on alkali-labile P) was about 50%. Fraction II was used.

These preparations are by no means completely pure; besides the inorganic phosphate, they contain some phosphoglyceric acid, formed by the iodine-oxidation of glyceraldehyde phosphate, and not completely removed by the alcohol fractionation. There is also probably present an unknown decomposition or condensation product which is enzymatically inactive, but which does give alkali-labile P and also methylglyoxal; estimations of alkali-labile P and methylglyoxal indicate that there should be present 10% more triose phosphate than actually reacts enzymatically in the spectrophotometer.

Contamination by glyceraldehyde phosphate is easily detected spectrophotometrically by the jump in the absorption when the dihydroxyacetone phosphate preparation is added to the oxidizing system, in the absence of isomerase. The preparation used here contained not more than 0.5% of glyceraldehyde phosphate.

It is probable that further investigation would reveal the nature of all the impurities, and that methods of fractional precipitation could be devised for removing them. However, since they do not interfere with the isomerase determination, no further efforts were made in this direction.

It may be remarked that an enzymatic synthesis of pure D-glyceraldehyde 3-phosphate is theoretically quite possible. If our ordinary triose phosphate were reacted with dihydrocozymase, in the presence of the glycerophosphate dehydrogenase of Baranowski (10), the dihydroxyacetone phosphate would be reduced to glycerophosphate, which could then be separated from the remaining glyceraldehyde phosphate. This presupposes the absence of triose phosphate dehydrogenase, and of isomerase. The necessity for stoichiometric amounts of dihydrocozymase could be avoided by constantly regenerating it with lactic acid and lactic dehydrogenase.

KINETICS OF THE ISOMERASE REACTION

Meyerhof and Beck (3) have defined one unit of isomerase (and aldolase and other enzymes reacting with phosphorylated substances) as the amount which metabolizes 1 mg. bound P in 1 min. at 38°. This is measured by the initial rate of the reaction.² Moreover, they found that the rate of attainment of equilibrium from the side of glyceraldehyde phosphate is consistent with the idea that the reaction is unimolecular in both directions. The theoretical curve for such a reaction would be given by

$$2.3 \log \frac{S - x_1}{S - x_2} = (k_1 + k_2) (t_2 - t_1), \quad [3]$$

where S is the equilibrium concentration of dihydroxyacetone phosphate, x_1 and x_2 the concentrations at times t_1 and t_2 , respectively, k_1 the unimolecular rate constant for the conversion of glyceraldehyde phosphate into dihydroxyacetone phosphate, and k_2 the rate constant for the reverse reaction. Their experimental data fit the theoretical curve, at least up to about $\frac{2}{3}$ of the equilibrium concentration of dihydroxyacetone phosphate. Since $k_1/k_2 = K_{\text{equilibrium}}$, it is possible to determine k_1 and k_2 individually from the rate of approach to equilibrium.

The method of the present paper makes it possible to examine separately the conversion of dihydroxyacetone phosphate into glyceraldehyde phosphate. The chief results of this study may be stated immediately, and will then be discussed in detail.

1. The reaction

dihydroxyacetone phosphate \rightarrow glyceraldehyde phosphate
is of the first order.

² This unit was employed when the kinetics of the isomerase reaction were not yet known. Since the reaction has now been found to be first order in both directions, the enzyme activity is better described in terms of the velocity constants.

2. The rate constant k_2 is approximately the same as that calculated from the rate of approach to equilibrium.
3. k_2 is proportional to the isomerase concentration, at least over the tenfold range of concentration where accurate measurement is possible.
4. The activity of the enzyme is the same at pH 7 as at 7.8, but decreases 50% at pH 6.3.
5. Aldolase does not affect the determination of isomerase activity.

EXPERIMENTAL RESULTS AND DISCUSSION

Figure 1 illustrates a typical spectrophotometer curve. There is a minute increase in density when the dihydroxyacetone phosphate is

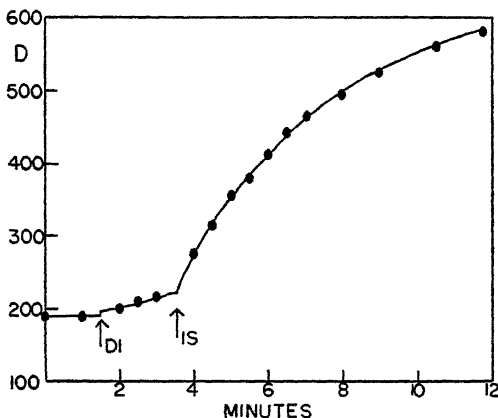


Fig. 1. Action of isomerase measured by the Beckman spectrophotometer. Ordinate: D , density; abscissa, time in minutes. \uparrow DI: 0.22 μ moles dihydroxyacetone phosphate added (the minute step in the curve corresponds to a contamination with 1/200 glyceraldehyde phosphate). \uparrow IS: 0.05 ml. isomerase added. For details, see text.

added; this is due to the oxidation of the small amount of glyceraldehyde phosphate present (0.5%) as an impurity in the dihydroxyacetone phosphate. After this glyceraldehyde phosphate is consumed, there is a slow but constant increase in density caused by traces of isomerase in the dehydrogenase, which maintain a steady trickle of glyceraldehyde phosphate into the oxidizing system. The main portion of the curve begins with the addition of isomerase, and represents a first-order reaction (cf. Fig. 2).

The rate constant k_2 is best obtained by drawing the best possible curve through the experimental points (as in Fig. 1), and then calculating k_2 from points on this curve (besides the initial point) as follows:

For a first order reaction,

$$2.3 \log \frac{c_0}{c} = k_2 t, \text{ or } 2.3 \log \frac{d_x - d_0}{d_x - d} = k_2 t, \quad [4]$$

where d_x , d_0 , and d are the densities at $t = \infty$, $t = 0$, and t , respectively. For two different times,

$$2.3 \log \frac{d_x - d_0}{d_x - d_1} = k_2 t_1 \text{ and } 2.3 \log \frac{d_x - d_0}{d_x - d_2} = k_2 t_2 \quad [5]$$

can be solved for d_x and k_2 . The term $d_x - d_0$ measures the total dihydroxyacetone phosphate, so that the method can be used for the analysis of dihydroxyacetone phosphate; k_2 measures the enzyme activity. The curve in Fig. 2 has been drawn in this way, using the observed d_0 (0.220) and the calculated values of d_x (0.623) and k_2 (0.253/min.).

A small correction may be made for the isomerase in the glyceraldehyde phosphate dehydrogenase. Figure 1 shows that the isomerase in the dehydrogenase supplies glyceraldehyde phosphate at a rate equivalent to a density increase of 0.013/min., at the initial concentration of dihydroxyacetone phosphate. Since $dc/dt = kc$, we have $0.013 = k(0.623 - 0.220)$, whence $k = 0.032/\text{min}$. This figure is subtracted from the total k_2 value, giving a net k_2 of 0.221/min.

The present method is applicable only to purified isomerase preparations, including acetone powders. Crude tissue extracts contain the glycerophosphate dehydrogenase of Baranowski (10) which causes the reduced DPN to react with the dihydroxyacetone phosphate. When a specific poison for this dehydrogenase is found, the method will become useful for all tissues and extracts, except those containing large amounts of phosphatase or DPN-destroying enzymes.

It was found that most of this dehydrogenase could be removed by precipitation in 36.4% ammonium sulfate (36.4 g./100 ml. solution) which does not precipitate isomerase. However, some dehydrogenase activity remained to lower the apparent isomerase activity. An attempt to inactivate the dehydrogenase with mercuric nitrate was also only partially successful.

In view of this situation it is of both theoretical and practical interest to know whether the k_2 value obtained spectrophotometrically is the same as that found from the rate of approach to equilibrium from the side of glyceraldehyde phosphate. Accordingly, the enzyme used to obtain the curve in Fig. 1 (k_2 (corr.) = 0.22) was also employed for a determination by the iodine oxidation method. The initial percentage of dihydroxyacetone phosphate in the triose phosphate was 32, and the

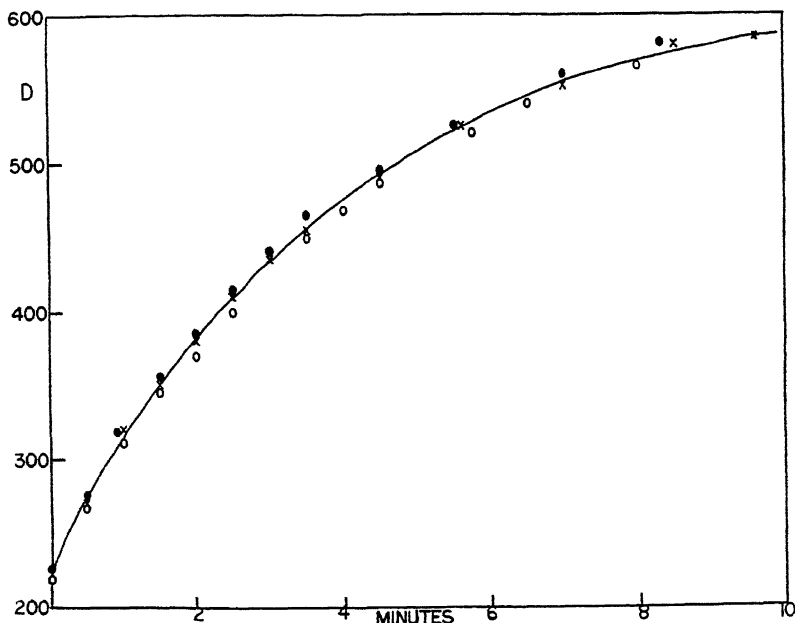


FIG. 2. First-order curve for isomerase ($k = 0.253$). Density vs. time. Solid curve is theoretical. Two experiments with isomerase alone (● and ○), and one with isomerase and aldolase (×).

equilibrium percentage is 95. Substitution in [3] gave $k_1 + k_2 = 0.46$, when the reaction was carried to 55% dihydroxyacetone phosphate, or 0.35 when the reaction was carried to 64% dihydroxyacetone phosphate. (The decrease in rate as the equilibrium is approached was also found by Meyerhof and Beck.) Since k_2 is 5% of $k_1 + k_2$, $k_2 = 0.023$ (or 0.018); since the enzyme was diluted eight times, compared with that used in the spectrophotometric determination (because the speed is much

greater from the side of glyceraldehyde phosphate), we obtain, finally, $k_2 = 0.184$ (or 0.144) for comparison with 0.22.

The agreement is as close as can be expected in view of the necessary inaccuracy of the iodine method. Furthermore, the ratio of k_2 to $k_1 + k_2$ is very sensitive to the correct equilibrium percentage of dihydroxyacetone phosphate. A change of 1% in this percentage would shift the calculated value of k_2 by 20%.

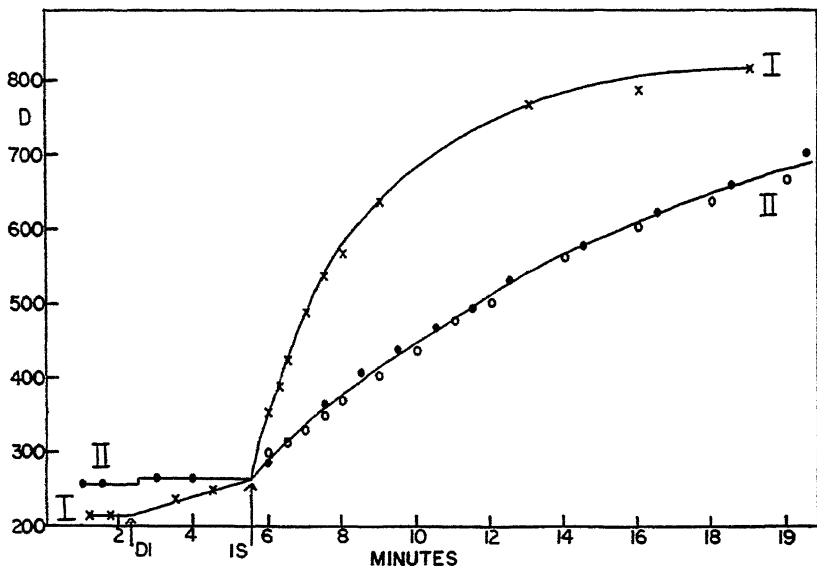


FIG. 3. Effect of phosphate on isomerase action. Density vs. time. All experiments with 0.33 μ moles dihydroxyacetone phosphate and 0.05 ml. isomerase.

Curve I, \times , no phosphate added; curve II, \bullet , 0.05 M phosphate added; \circ , the same phosphate and double the amount of oxidizing enzyme, which has no effect on the speed.

\uparrow DI: dihydroxyacetone phosphate added. \uparrow IS: isomerase added. (The same moment for all three experiments.)

The activity of the same isomerase solution was also tested by Warburg's first spectrophotometric method. This method likewise measures the rate of attainment of equilibrium from the side of glyceraldehyde phosphate, so that [3] should be applicable. We found, however, that isomerase is very sensitive to inorganic phosphate and that at a concentration of 0.05 M , which we used in accordance with Warburg and

Christian, isomerase reacts with the speed of only one-fourth of that in the absence of phosphate (see Fig. 3). With still higher concentrations of phosphate the speed is lowered still more. While k_2 with our method was 0.21, it was 0.026 with 0.165 *M* phosphate and 0.047 with 0.082 *M* phosphate.

The effects of pH, isomerase concentration, and presence of aldolase are shown in Table I. The ratio k_2/c should be constant throughout for

TABLE I
Velocity Constants of Isomerase Under Various Conditions

| Isomerase preparation no. | Method ^a | pH | Isomerase units | k_2 | $k_2/\text{conc.}$ |
|---------------------------|---------------------|-----|-----------------|-------|--------------------|
| 1 | Spectr. | 6.3 | .76 | .16 | .21 |
| | | 6.3 | 1.46 | .27 | .18 |
| | | 6.3 | 3.82 | .68 | .18 |
| | | 7.0 | .39 | .16 | .41 |
| | | 7.0 | .73 | .27 | .37 |
| | | 7.0 | 3.82 | 1.23 | .30 |
| | | 7.8 | .39 | .16 | .41 |
| | | 7.8 | .76 | .28 | .37 |
| | | 7.8 | 3.82 | 1.50 | .39 |
| | | | | | |
| 2 | | 7.0 | .54 | .20 | .37 |
| | | 7.0 | 1.08 | .40 | .37 |
| 3 | | 7.0 | .57 | .21 | .37 |
| | | 7.0 | 1.13 | .42 | .37 |
| 4 + aldolase | Iodine ^a | 7.0 | .59 | .22 | .37 |
| | | 7.0 | .59 | .16 | .27 |
| | | 7.0 | 1.17 | .38 | .32 |
| | | 7.0 | .59 | .23 | .39 |
| | | | | | |

^a Spectrophotometric in all cases but one.

the same pH. This is actually the case, as the last column of Table I shows.

Attention may be called to the absence of any effect of aldolase (Prepn. 4). Enough aldolase was added to the cuvette to have formed the total triose phosphate present in 1.5 min. from hexose diphosphate (HDP).

The isomerase contents of a number of tissues are given in Table II. The determinations by the iodine method were all made on the supernatants from tissue homogenates, except in the case of yeast where the cells in a suspension were disrupted by ultrasonic vibration, and the debris centrifuged out.

As one can see from the last two columns in Table II isomerase is always about 40–200 times in excess over aldolase, measured in the same way and for roughly the same concentrations.

TABLE II
Isomerase Units in Various Tissues
All values per gram fresh tissue (38°)

| No. | Tissue | ($k_1 + k_2$) | k_1 | k_2 | Isomerase units (from k_1) | Aldolase units | Ref. |
|-----|------------------------------|-------------------|-------|-------|-------------------------------|----------------|------|
| 1 | Brain | 789 | 750 | 39 | 105 | 0.34 | (11) |
| 2 | " | 505 ^a | 479 | 25 | 67 | | |
| 3 | Yeast | 5600 ^b | 5320 | 280 | 745 | 15.6 | |
| 4 | Rat sarcoma ^c | 504 ^c | 478 | 25 | 67 | 0.6 | (11) |
| 5 | Mouse tumor | 264 | 251 | 13 | 35 | — | |
| 6 | Angioma from pituitary (rat) | 310 ^d | 294 | 16 | 41 | 0.3 | |
| 7 | Muscle | 2150 | 2050 | 107 | 288 | 7.5 | (11) |

^a One g. tissue gives 141 mg. protein in the homogenate, biuret method (12).

^b One g. fresh bakers' yeast gives 95 mg. protein in the ultrasonic solution.

^c One g. tissue gives 126 mg. protein in the homogenate.

^d One g. tissue gives 133 mg. protein in the homogenate.

^e We thank Dr. Margaret Lewis, Wistar Institute, Philadelphia, Pa., for the various tumors.

It follows from our investigation that for practical purposes with tissue extracts the chemical method which was developed formerly and which is based on the disappearance of glyceraldehyde phosphate is the only feasible one, because for the isomerase the formula $K(\text{equil.}) = k_1/k_2 = 0.045$ can be applied. This allows one to calculate the speed in the opposite direction also, which is biologically more important. The reaction studied spectrophotometrically with purified isomerase obeys the mass-action law for a first-order reaction. The apparent disagreement of this speed with that measured by the procedure of Warburg and Christian disappears when the high sensitivity of isomerase to phos-

phate is taken into account. In 0.05 *M* phosphate the speed is only a fourth of that in the absence of phosphate.

SUMMARY

The isomerase reaction was studied in both directions. With purified isomerase starting from the side of dihydroxyacetone phosphate, the reaction can be followed spectrophotometrically by applying the principle of O. Warburg, coupling the reaction with the oxidation of glyceraldehyde phosphate by cozymase. The reaction is of the first order and it can be shown that the equation $K_{eq} = k_1/k_2 = 0.045$ can be applied.

This method is, however, unsuitable for tissue extracts because of interfering enzymes. On the other hand, the spectrophotometric measurement from the side of glyceraldehyde phosphate in the presence of phosphate gives no absolute results because the isomerase is highly sensitive to inorganic phosphate. Therefore, only the chemical determination of the disappearance of D-glyceraldehyde 3-phosphate can be applied. In this way it can be shown that not only in muscle but also in brain, malignant tumor, and yeast, the activity of isomerase is about 40–200 times as great as that of aldolase if the speed is measured under similar conditions.

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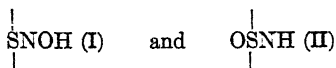
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LETTERS TO THE EDITORS

Studies on Nitrogen Trichloride-Treated Prolamines.

VIII. Synthesis of the Toxic Factor

The toxic factor found in nitrogen trichloride-treated flour (1) and in certain proteins similarly treated (2,3) was isolated recently from nitrogen trichloride-treated zein (4,5) and flour (6). The compound analyzed correctly for $C_5H_{12}N_2O_3S$ and was found to be a derivative of methionine and a methionine antagonist (5,7,8,9,10). Evidence was also presented (7,11) indicating that the additional atoms of H, N, and O in the toxic factor were attached to the sulfur. This was further supported in our laboratories by the conversion of the toxic factor to methionine sulfone by oxidation with 30% hydrogen peroxide in acetic acid for 6 days at 40 C. The structures



although not encountered before, appeared to be more likely than others considered. Structure II was supported by showing that acid hydrolysis of the toxic factor produced ammonia but no hydroxylamine. The infrared spectrum¹ of the compound also tended to favor II.

Numerous attempts to prepare the compound from methionine and from some of its derivatives by treating them with nitrogen trichloride failed. However, we succeeded in synthesizing II by reacting methionine sulfoxide with hydrazoic acid. A crude product isolated from the reaction mixture contained a chromatographic component which had the same R_f values as the toxic factor. Furthermore, it produced typical seizures in rabbits. Purification of the product yielded needle-shaped crystals which melted with decomposition in the same range as those of the toxic factor isolated from zein and were in appearance and on paper chromatograms indistinguishable from the latter. The synthetic compound produced typical seizures in mice at a dose of 100 mg./kg. intravenously but not at 50 mg./kg. Seizures did not develop when 2 g./kg. of methionine was administered intraperitoneally at the same time. The minimum convulsive dose in rabbits was about 3 mg./kg. Thus the toxicity is about the same as that of most batches of toxic factor obtained from treated zein although some batches were considerably more toxic. This is, however, not surprising since D,L-methionine was used in the synthesis whereas the toxic factor in zein was derived from L-methionine and might have been racemized to a different degree in different runs. Since the reaction of a sulfoxide with hydrazoic acid has not been reported before and its mechanism has not yet been studied, the successful synthesis of the toxic factor by this new reaction cannot be taken to prove structure II conclusively.

EXPERIMENTAL

Methionine sulfoxide (3.3 g.; 0.02 equivalent) prepared from D,L-methionine (12) was placed in a three-necked flask equipped with stirrer, thermometer, and dropping

¹ Carried out by Dr. Richard Lord, M. I. T.

funnel. Twenty ml. of chloroform and 5 ml. of sulfuric acid (96%) were added first, then 30 ml. of 1 *N* hydrazoic acid in chloroform (13) was added over a period of 1 hr. The temperature was kept at 43–46°C. and the mixture was stirred at the same temperature for an additional 3 hr. It was then poured onto crushed ice and the aqueous layer was treated with enough anion exchanger (IR-4B) to bring the pH to 8. The hydrogen-ion concentration was then adjusted to pH 4 with a cation exchanger (Duolite-Cl) and the solution was concentrated to 25 ml. *in vacuo*. Ethanol (125 ml.) was then added and the precipitate formed was extracted with 50 ml. hot water, the extract concentrated to 10 ml. and mixed with 3 ml. methanol. Prism-shaped crystals deposited in a few hours; when recrystallized from water they had the characteristic needle shape. The compound began to decompose at 220°C. and melted within the same range (230–8°C.) as most samples of the compound isolated from zein. Mixed samples did not show a depression of the melting point. *R_f* values (ascending): 0.05, butanol-acetic acid; 0.62, phenol; and 0.27, lutidine-collidine-ethanol.

Elementary Analysis: Calculated: C, 33.32; H, 6.57; N, 15.55; S, 17.79. Found: C, 33.37; H, 6.79; N, 15.65; S, 17.92.

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The Purification and Crystallization of Malt β -Amylase

The crystallization of sweet potato β -amylase (1) and of malt α -amylase (2) has been described by Balls *et al.*, while in our laboratory α -amylases of swine (3,4) and human (5) pancreas, of human saliva (6), and of *Bacillus subtilis* (7), have been isolated in crystalline form. We now report the crystallization of the β -amylase of malt.

Our starting material was the malt extract "Diastafor" supplied by Wander and Co. of Berne, Switzerland. This preparation contains a mixture of α - and β -amylases, salts, and about 30% of sugar and polysaccharides. It was purified as follows:

Elimination of α -Amylase

An aqueous 1:3 suspension of Diastafor was left for 30 min. at pH 3.6 (acetic acid), and then filtered with the aid of "Filter-cell."

Purification of β -Amylase

The filtrate was brought to pH 5, solid ammonium sulfate was added to 0.6 saturation and the mixture was then passed through a Sharples supercentrifuge. The liquor, which contained practically all the sugars and polysaccharides, was rejected. The precipitate was extracted with water, in which the enzyme was soluble, and the solution fractionated twice by acetone at pH 5. The fraction precipitating between 37–60% acetone was first taken, and then that between 40–50%. This product was dissolved and brought to pH 7.8 (0.1 N/ammonium acetate + ammonia) when, on addition of acetone, the fraction precipitating between 51–56% was found to be pure β -amylase. Electrophoresis at pH 3.6, 3.9, 7.0, and 7.9 showed in all cases only one component. Over the whole purification process the activity yield is about 10% with an activity/nitrogen enrichment of about fifteen times with respect to the original Diastafor.

Fig. 1. Crystalline malt β -amylase.

Crystallization and Recrystallization

Ammonium sulfate was added to a 4–5% solution at pH 6.3 until the first precipitate appeared; the solution was then left over silica gel at 5°C. After 15 hr. crystals began to appear. The suspension was centrifuged after standing for 8 days, the crystals were dissolved in a minimum quantity of water and retreated in the same manner (Fig. 1).

Properties

The crystalline enzyme has a specific activity of 1660 mg. maltose/mg. N (8). It contains neither sugars nor polysaccharides, and has a nitrogen content of 14.1%. Its solubility in water is much greater than that of the other amylases. The activity is not dependent upon the presence of chloride ions as for the animal, bacterial, and

taka-amylases, nor does it need calcium as does malt α -amylase. The enzyme may be dialyzed without loss of activity against buffer solutions at various pH values. It may be easily and irreversibly destroyed at its isoelectric point which is $\text{pH } 6 \pm 0.1$, although it is otherwise stable between pH's 4 and 8. The enzyme is sharply differentiated from sweet potato β -amylase because it is irreversibly deactivated by heavy-metal ions which do not affect the latter.

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The Enzyme-Catalyzed Exchange of Ammonia With the Amide Group of Glutamine and Asparagine¹

The occurrence of enzymes which catalyze the exchange of the amide group of two biologically important amides—glutamine and asparagine—with hydroxylamine was recently demonstrated (1). Cell-free extracts of *Proteus vulgaris* X 19 are a particularly potent source of the enzymes.

It does not seem likely that the formation of hydroxamic acids is the only function of the enzyme system; it is more probable that hydroxylamine is a substitute for amines of biological significance, e.g., amino acids, and that the enzyme system plays a role in peptide synthesis. The naturally occurring amides may, therefore, be visualized as stores of energy approximating that of a peptide bond.

Since the enzymatic formation of γ -glutamo- and β -aspartohydroxamic acid from the corresponding amides may be considered as a model for other exchange reactions, the reactivity of the amide groups in an enzyme catalyzed exchange with ammonia, labeled with N^{15} , was tested. Glutamine, asparagine, and labeled ammonium chloride were incubated with a cell-free extract of *Proteus vulgaris* for 90 min. Control experiments were carried out without the addition of enzyme extract. After the removal of the proteins with trichloroacetic acid, the protein-free filtrates were distilled *in*

¹ Supported by grants from the Rockefeller Foundation and the National Vitamin Foundation. We are indebted to Dr. D. Rittenberg and Mr. Sucher whose cooperation made this study possible.

vacuo at pH 10 to remove the labeled ammonia. In order to dilute any traces of remaining isotope, nonlabeled ammonium chloride was added three times and the ammonia was removed *in vacuo*. The amide groups were liberated by acid hydrolysis and the ammonia was collected by distillation after the solution had been made alkaline. In Table I the isotope content of the ammonia of the last distillate before the liberation of the amide groups is given as a footnote.

TABLE I
*Enzymatic Exchange of Ammonia with the Amide Groups of
Glutamine and Asparagine*

| Addition | Enzyme extract | | Present |
|-------------------------|-----------------------------------|----------|----------|
| Absent | N^{15} found in amide group | | Exchange |
| μmoles | Excess ^a atom— $\%$ | | per cent |
| Glutamine ^a | | | |
| 120 | 0.118 (1) | 1.88 (3) | 5.0 |
| NH_4Cl^d | | | |
| 260 | | | |
| (Glutamine ^b | | | |
| 240 | | 2.74 (4) | 7.7 |
| NH_4Cl^d | | | |
| 680 | | | |
| Asparagine ^c | | | |
| 300 | 0.018 (2) | 1.39 (5) | 4.3 |
| NH_4Cl^d | | | |
| 500 | | | |

^a Three ml. enzyme solution or water, total volume 12 ml.

^b Nine ml. enzyme solution, total volume 20 ml.

^c Four-tenths ml. of enzyme solution or water, total volume 4 ml. Enzyme solution contained 1 mg. undialyzable N/ml.

^d Thirty-two atom-3% excess N^{15} .

N^{15} atom-% excess in NH_3 of last distillate before liberation of amide group: (1) 0.235, (2) 0.040, (3) 0.296, (4) 0.275, (5) 0.018.

In the experiments in which the amide was incubated with isotopic ammonia without enzymes, the apparent isotope concentration of the amide group was below that of the ammonia of the last distillate before liberation of the amide group. However, in the presence of enzymes a considerable exchange took place. Up to 8% of the amide group of glutamine was exchanged in an experiment of 90 min. duration.² The

² It was reported recently that papain catalyzes the exchange of ammonia with the amide groups of synthetic substrates. [FRUTON, J. S., *Yale J. Biol. and Med.* **22**, 263 (1950).]

rapid exchange of the amide groups of asparagine and glutamine with ammonia supports the view that amides, once formed—probably by mediation of energy-rich acyl phosphate (2)—may take part in further synthetic reactions by transfer of the glutamyl or aspartyl radical.

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 $C^{14}O_2$ Assimilation by Soybean Leaves¹

We wish to summarize our findings on the photosynthetic assimilation of $C^{14}O_2$ by excised soybean leaves during relatively short periods (5–90 sec.).

Following the assimilation period and the removal of the residual $C^{14}O_2$, the leaves were killed by immersion in liquid nitrogen or CO_2 -80% aqueous ethanol. The total time from conclusion of the experiment to leaf killing never exceeded 10 sec. The nature of the products, as shown by chromatography, was not materially changed by the temperature of the extraction with 80% ethanol, *i.e.* at 0°C., room temperature, reflux temperature.

The primary chromatographic product of carbon dioxide fixation is free glyceric acid. Phosphoglyceric acid increases in relative significance with shorter time intervals, although at the shortest time (5 sec.), it is still considerably less radioactive than the non-phosphorylated acid.

The second most prominent product is alanine. Significant, but substantially smaller amounts of glycine and serine are present, the latter being of higher specific activity than the former. No radioactive aspartic acid was detected within the period of investigation. These results are, except for the degree of phosphorylation, not significantly different from those summarized by Benson and Calvin (1).

Particular attention has been given to the sequence of carbohydrate formation, with the following conclusions arrived at.

1. The first free sugars formed are the trioses and glucose. It is probable that the trioses precede the glucose, if extrapolations of relative specific activities are made to zero time. At the shortest time intervals used in these studies (15 sec.) their specific activities were approximately equal. Subsequent to these sugars are formed sucrose, then raffinose and mannose, and possibly fructose. (We are not certain of the extent to which the observed free fructose arises from hydrolysis of sucrose during in ex-

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¹ This work was performed in part in the Ames Laboratory of the Atomic Energy Commission.

change.) In lengthy assimilation (15 min.), the sugar present in largest concentration, as well as highest specific activity, is sucrose. Glucose, though in higher concentration than raffinose, is of lower specific activity, while mannose, of a concentration approximately equal to that of glucose, is the least radioactive of the four. Free fructose is present only in very minor quantities. (By contrast, in the roots of these plants the primary sugar is fructose, and the first and main product of phosphorylation is fructose 1,6-diphosphate.)

2. Under all conditions the free hexoses and those obtained from the disaccharides are composed of two equal halves. Because of the nonuniformity of radioactivity within each half of the glucose, that compound must be derived from two 3-C units, probably the trioses.

3. The glucose portion of the sucrose is formed prior to the fructose. The fructose from the hydrolyzed sucrose shows almost uniform distribution of activity among the six carbons at all times, while glucose from the same does not. Thus the formation of sucrose appears to follow a pathway different from that of glucose.

This sequence of carbohydrate formation is not in agreement with that suggested for *Chlorella* (2), but there is no reason to believe that an identical pathway is required for all plants.

A full exposition, including experimental details, will be published shortly.

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The Quantitative Crystalline Vitamin B₁₂ Requirement of the Baby Pig

Johnson and Neumann (1), using a "synthetic" milk ration with an isolated soy-bean protein (alpha protein) as the nitrogen source, have reported that the baby pig requires vitamin B₁₂. Essentially the same basal ration was used in this study of the quantitative vitamin B₁₂ requirements of the baby pig. Two experiments were conducted to determine (I) the vitamin B₁₂ requirement when given by intramuscular injection, and (II) the oral vitamin B₁₂ requirement and the relationship of the injected to the oral requirement.

In Expt. I, 14 two-day-old pigs received the basal ration for a 2-week depletion period, after which they were allotted to five groups and individually fed for 6 weeks. The amounts of crystalline vitamin B₁₂ injected intramuscularly per pig daily, with the results, are given in Table I.

In Expt. II, 15 two-day-old pigs were allotted to five groups and individually fed the same basal ration as in Expt. I for 6 weeks. The amounts of crystalline vitamin B₁₂ fed orally to Groups 1 through 4 and the amount of vitamin B₁₂ injected intramuscularly in Group 5, with the results, are given in Table II. The average daily gain of Group 3 was significant over the average daily gains of Groups 1 and 2 ($P > 0.01$).

TABLE I

Response of Baby Pigs to Injected Levels of Crystalline Vitamin B₁₂

| | Group 1 Basal | Group 2 Basal + 0.1 µg. B ₁₂ /kg. body weight/day | Group 3 Basal + 0.2 µg. B ₁₂ /kg. body weight/day | Group 4 Basal + 0.4 µg. B ₁₂ /kg. body weight/day | Group 5 Basal + 0.6 µg. B ₁₂ /kg. body weight/day |
|---|------------------|---|---|---|---|
| No. of pigs | 2 | 3 | 3 | 3 | 3 ^a |
| Av. initial weight, kg. (2-week-old) | 2.28 | 2.22 | 2.30 | 2.31 | 2.65 |
| Av. final weight, kg. | 4.86 | 10.39 | 14.49 | 14.34 | 19.73 |
| Av. daily gain, lb. | 0.14 | 0.43 | 0.64 | 0.63 | 0.90(0.82) ^{b,c} |
| µg. B ₁₂ injected/kg. dry matter con- sumed (av. for 6-week period) | 0 | 1.58 | 3.13 | 5.22 | 10.15 |

^a One pig developed a severe diarrhea during the fourth week that retarded his growth. From observation of the pigs it was evident his behavior was not typical of the group.

^b Average daily gain of all (3) pigs in the group.

^c The average daily gain of Group V was significant over the average daily gain of each of the other groups (Group 5 vs. Group 4, $P = .04$; Group 5 vs. Group 3, $P > .01$).

TABLE II

*Response of Pigs to Oral Levels of Crystalline Vitamin B₁₂
as Compared with Injected Level*

| | Group 1 10 µg. B ₁₂ kg. dry matter consumed | Group 2 15 µg. B ₁₂ / kg. dry matter consumed | Group 3 20 µg. B ₁₂ / kg. dry matter consumed | Group 4 40 µg. B ₁₂ / kg. dry matter consumed | Group 5 0.6 µg. B ₁₂ injected/kg. body wt./day |
|-------------------------|--|--|--|--|--|
| No. of pigs | 3 | 3 | 3 ^a | 3 | 3 |
| Av. initial weight, kg. | 1.79 | 1.73 | 1.82 | 1.80 | 1.71 |
| Av. final weight, kg. | 11.79 | 11.57 | 17.31 | 17.46 | 16.96 |
| Av. daily gain, lb. | 0.53 | 0.51 | 0.82 ^b | 0.82 ^b | 0.80 ^b |

^a One pig was removed from the group due to an infection that persisted for 3 weeks. After recovery from the infection this pig gained at the rate of 1.05 lb./day, which was comparable to the other pigs of the group. When this pig is included in the average of the group, the average daily gain of Group 3 approaches significance over Groups 1 and 2 (odds, 17:1).

^b Highly significant over average daily gains in Groups 1 and 2 ($P > 0.01$).

The average daily gains of Groups 3, 4 and 5 taken together were highly significant over the average daily gains of Groups 1 and 2 taken together ($P = 0.0004$).

The oral vitamin-B₁₂ requirement based on these results is approximately 20 $\mu\text{g.}/\text{kg.}$ of dry matter consumed. The requirement by injection is approximately 0.6 $\mu\text{g.}$ vitamin B₁₂/kg. of body weight daily. This is approximately half of the oral requirement.

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Conjugation of Phenol by Rat Liver Homogenate¹

We reported some years ago (1,2) that phenol is conjugated by slices of liver and other tissues of the rat and other animal species. We also found (2) that liver "brei" was unable to conjugate phenol. Our observations (2) led us to believe that a source of energy was required for this conjugation and this was probably provided by a coupled oxidation. After many unsuccessful attempts, we finally arrived at a set of conditions that have permitted us to obtain reproducible results with rat liver homogenate.

A 10% homogenate is prepared by dropping fresh liver in the medium (described below) contained in a modified semimicro monel-metal jar of the Waring Blendor (a jacket had been added to the jar to circulate ice water). The complete medium placed

TABLE I
Conjugation of Phenol by Rat Liver Homogenate

| Phenol | α -Ketoglutarate | Adenylic acid | Sulfate | Free phenol | Bound phenol |
|--------|-------------------------|---------------|---------|------------------|----------------|
| | | | | $\mu\text{g.}$ | $\mu\text{g.}$ |
| + | + | + | + | 19 | 112 |
| + | — | — | + | 147 | 4 |
| + | + | — | + | 153 | 4 |
| + | — | + | + | 142 | 3.5 |
| + | + | + | — | 112.5 | 8 |
| + | + | + | + | 142 ^a | — |

^a Control not incubated.

in the jar had the following composition: 100 ml. 0.154 *M* NaCl; 4 ml. 0.154 *M* KCl; 3 ml. 0.154 *M* MgSO₄; 21 ml. 0.1 *M* sodium phosphate buffer pH 7.4; 0.532 mmoles phenol; 0.01 *M* α -ketoglutaric acid, and 0.001 *M* adenylic acid. The final pH is adjusted to 7.4. The liver is homogenized for 30 sec. in the Blendor. It is then immediately transferred to an all-glass tissue crusher of the Ten Broeck type, and crushed

¹ This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

until homogenation is complete. The preparation is maintained at a temperature of 0–5°C. throughout the processing and before incubation is started. In each series of experiments two control samples were taken before incubation, the proteins precipitated with tungstic acid, and phenol determined by a method described previously (3). All vessels were gassed with oxygen and incubated for 4 hr. at 37.5°C. with 100 shakings/min. At the end of incubation, proteins were precipitated with tungstic acid, and phenol determined in the supernatant. The bound phenol was determined after 15 min. hydrolysis with 0.3 *N* HCl in a boiling water bath, which gives phenol bound to sulfate.

The results obtained are indicated in Table I.

It is evident that the presence of both adenylic and ketoglutaric acids is indispensable for this conjugation in the liver homogenate. When magnesium chloride was substituted for magnesium sulfate there was a considerable decrease in bound phenol, indicating that most of the phenol was bound to sulfate when the latter was present.

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A Labeled Carcinogenic Azo Dye; 3'-Methyl (C¹⁴)-4-Dimethylaminoazobenzene

Several groups of investigators (1) have synthesized and tested many different azo compounds for carcinogenic activity. Miller and Baumann (2) in 1945 reported that the compound 3'-methyl-4-dimethylaminoazobenzene, which differed from 4-dimethylaminoazobenzene by an additional methyl group, was an extremely active carcinogenic agent. The addition of this methyl group increased the carcinogenic activity two to three times over that of the parent structure.

From this it would appear that the methyl group in the 3'-position of the dye molecule may have an important role in directing the course of liver cancer development. In order to facilitate a study of the mechanism of azo dye carcinogenesis, the synthesis of 3'-methyl (C¹⁴)-4-dimethylaminoazobenzene was undertaken.

EXPERIMENTAL

The experimental procedure for the synthesis of the C¹⁴-labeled dye was as follows: 11 g. of bromobenzene was reacted with 1.6 g. of magnesium to form the Grignard reagent, phenylmagnesium bromide, which was then exposed to carbon dioxide containing 1.0 millicurie of C¹⁴O₂, resulting in carboxyl-labeled benzoic acid. These reactions were carried out in a low pressure, all-glass system described by Dauben and associates (3). The benzoic acid was esterified with methyl alcohol and nitration was then effected according to the method of *Organic Syntheses* (4) resulting in 5.4 g. of *m*-nitromethyl benzoate with an over-all yield of 50%; the total activity of the ester

was 0.5 millicurie. Considerable difficulty was encountered in preliminary attempts to reduce the *m*-nitromethyl ester to *m*-toluidine. Standard methods of reduction, including high-pressure hydrogenation failed to give the desired product. The reaction was finally accomplished by means of a new synthetic method using hydrazine as the reducing agent (5).

To 2.0 g. of the *m*-nitromethyl ester in 12 ml. of diethylene glycol, were added 3 ml. of 85% hydrazine and 2 g. of potassium hydroxide in concentrated aqueous solution. After a 10-min. period of refluxing, steam distillation resulted in a 30% yield of *m*-toluidine. The amine was diazotized and coupled with dimethylaniline, according to the standard procedure for azo-dye synthesis (2), which resulted in 0.4 g. of 3'-methyl-4-dimethylaminoazobenzene containing 0.2 millicurie of C^{14} activity.

Studies are in progress to determine the metabolism of this labeled carcinogenic agent.

ACKNOWLEDGMENTS

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We acknowledge with thanks the interest of Professor J. Murray Luck.

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Can β -Amylase Be Obtained from Clarase?

In the recent article by Lane and Williams (1) in which they reported the existence of inositol in pancreatic α -amylase, they used for comparative purposes a preparation from Clarase designated as β -amylase which is probably not β -amylase but rather a partially inactivated α -amylase.

The available evidence in the literature indicates that Clarase, which is a fungal amylase made from *Aspergillus oryzae*, is an α -amylase free from β -amylase, although it does contain other glucosidases. Caldwell and Adams (2) state that in repeated fractionation of fungal amylase no evidence was found for the concentration of a saccharogenic amylase component like that obtained in the separation of β -amylase from extracts of malted barley. Caldwell *et al.* (3) also state that there is no indication

of the presence of two amylases of widely different labilities such as the α - and β -amylases of malted barley. Redfern and Landis (4) show that the relative strength of fungal amylases is the same whether determined by the liquefying method, the Wohlgemuth method, or the method of Sandstedt *et al.* (SKB) (5). The liquefying and SKB methods are specific for α -amylase, but the presence of β -amylase would have been indicated, if present, by deviations in the Wohlgemuth method which is dependent on both α - and β -amylase (unless the β -amylase is in excess).

The method used by Lane and Williams for the preparation of " β -amylase" from Clarase is a method described by Hopkins, Murray, and Lockwood (6) for the preparation of β -amylase from barley. In this procedure the Clarase solution is acidified to pH 3.4, held at 0°C. for 24 hr., and then neutralized. The treated Clarase solution is then further purified by fractional precipitation with ammonium sulfate according to the method of Caldwell *et al.* (3). Although this method is quite satisfactory in differentially inactivating malt α -amylase, it will only partially inactivate fungal α -amylase because of the relative stability of fungal α -amylase at pH 3.4.

In order to study the relative stabilities of the two α -amylases, a 0.15% solution of Clarase 900 and a 20% malt infusion (clarified by centrifugation and filtration through a cotton plug) were adjusted to pH 3.4 at 0°C. with dilute acetic acid and stored at 0°C. in an ice bath. At intervals, aliquots were removed and analyzed by the Redfern (7) modification of the Sandstedt, Kneen, and Blish-Wohlgemuth method (5).

Table I shows the results obtained in this experiment. In 3 hr. 97.8%, and in 24 hr., 99.1%, of the malt α -amylase is inactivated. On the other hand, the activity of the Clarase α -amylase declined only 42.8% after 24 hr. Other experiments showed that over 90% of the malt α -amylase is inactivated in the first 30 min., whereas 30–40% of the Clarase α -amylase survives even after 48 hr. The difference in stability between these two α -amylases is quite marked and illustrates the difficulty in applying the same preparative methods to different classes of α -amylases. It was concluded that the preparation designated β -amylase by Lane and Williams must therefore be a partially inactivated α -amylase.

TABLE I

Relative Stability of Clarase and Barley Malt α -Amylases at pH 3.4 and 0°C.

| Enzyme solution | Time hr. | Decline in α -amylase potency per cent |
|-----------------|-------------|---|
| Clarase | 2 | 15.6 |
| | 24 | 42.8 |
| Barley Malt | 3 | 97.8 |
| | 24 | 99.1 |

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Xanthatin: An Antimicrobial Agent From *Xanthium pennsylvanicum*¹

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INTRODUCTION

It is now well-established that green plants are a promising source of new antimicrobial materials (1-3). While, in general, these factors have possessed a potency of a lower order of magnitude than antibiotic compounds isolated from microbial sources, they have occasionally proved useful as basic models for the synthesis of more active compounds (4) and in studies of mechanism of action (5).

This laboratory has been engaged in the isolation of such antibiotics possessing antifungal activity (6,7). Since insolubility in water is often considered to be a desirable property of fungicidal materials, our preliminary extractions are always made with absolute ethanol. One of the alcohol extracts demonstrating a pronounced antifungal activity was that of *Xanthium pennsylvanicum*, the cocklebur found along the sandy shore of Lake Champlain. Antimicrobial extracts have been reported from this species (8) and from *Xanthium americanum* (9). This report deals with the isolation, properties, and microbiological spectrum of the pure crystalline compound. Pending elaboration of the chemical structure, we have used the name "xanthatin" to designate this compound.

METHODS

Microbiological

Crude extracts which were used briefly for preliminary evaluation experiments were prepared by shaking weighed amounts of dried and ground material in measured

¹ Printed by permission of the Vermont Agricultural Experiment Station; Journal Series Paper No. 9. We are grateful to the Herman Frasch Foundation for a grant in support of this work.

volumes of absolute ethanol. The shaking was carried out for 2 hr. on an electrically-driven rotary shaker. The slurries were then filtered by suction and the alcohol extracts tested directly by cup-test procedures similar to those of Irving (10). Alcohol controls were always run simultaneously and no alcohol inhibition was found for any of the organisms used.

After a small quantity of pure crystalline material had been isolated using semi-quantitative cup-assay methods, a dosage-response curve (Fig. 1) was prepared and the plot used as a basis for the quantitative assay employed in developing the final solution procedure.

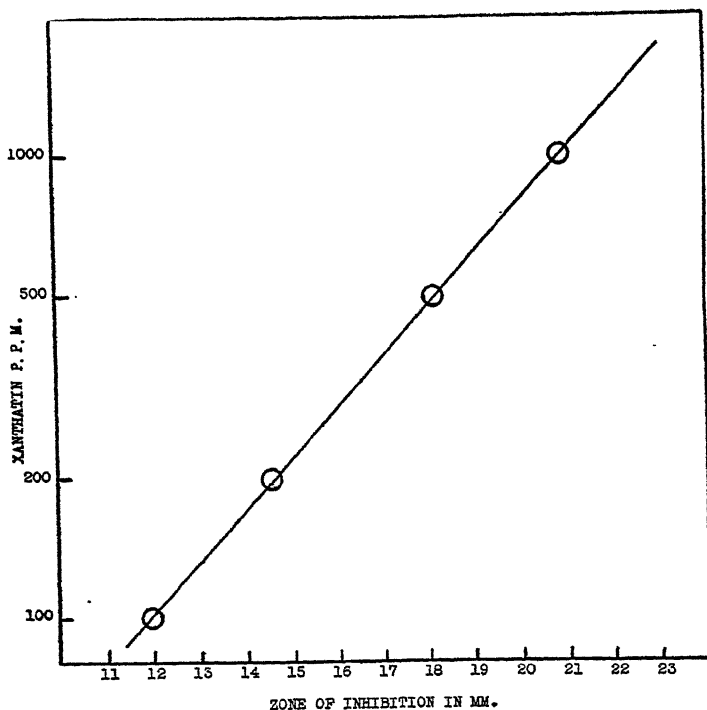


FIG. 1. Semilogarithmic dosage-response curve for the activity of an ethanol solution of pure xanthatin to the test organism *B. subtilis* by cup test.

EXPERIMENTAL

Isolation Procedure

After many trials the following procedure was developed. Four hundred g. of dried, ground leaves and burs of *Xanthium pennsylvanicum* were extracted by stirring for 2 hr. in 1000 ml. of benzene. The slurry was filtered and the cake washed on the funnel with a small additional quantity of benzene:

Fraction A.—Assay showed 598 mg. total xanthatin present. Total solids were 26,864 mg. Per cent purity = 2.2.

This benzene extract was run through a column of 700 g. of Fisher chromatographic alumina on a column 6 cm. in diameter, and the column was washed with enough additional benzene to remove a wide orange band. Three hundred ml. of ethyl ether was run through to remove more yellow material. These fractions were inactive.

The column was now eluted with acetone and 500 ml. of the yellow eluate collected:

Fraction B.—Assay showed 500 mg. total xanthatin present. Total solids were 8,900 mg. Per cent purity = 5.6.

Fraction *B* was concentrated to dryness *in vacuo* and the residual orange oil taken up in 60 ml. of benzene. This was run through a column prepared from 100 g. of Fisher alumina. No discrete bands were observable so the color was slowly washed through with benzene and collected arbitrarily as follows:

Fraction C.—(First colored fraction) total volume was 100 ml. Assay showed 192 mg. of pure xanthatin. Total solids were 600 mg. Per cent purity = 32.0.

Fraction D.—(Second colored fraction) total volume was 200 ml. Assay was 176 mg. Total solids were 800 mg. Per cent purity = 22.0.

Fraction E.—(Third colored fraction) total volume was 100 ml. Assay was 40 mg. Total solids were 340 mg. Per cent purity = 11.8.

Further addition of benzene and ether washed off a small quantity of very impure material. Fractions *C*, *D*, and *E* were combined and concentrated to dryness *in vacuo*. Fifteen ml. of Skellysolve B was added to dissolve a yellow contaminating oil, and the suspension was filtered through a sintered glass funnel. Impure crystals amounting to 472 mg. were obtained. Recrystallization was carried out by dissolving in hot methanol, adding water until the solution became cloudy, and cooling in ice. Two more crystallizations of this type, the last combined with a hot norite filtration, yielded 317 mg. of pure white crystalline material melting at 114.5–115.0°. A total recovery of 53% was obtained.

Properties

The compound when completely pure crystallizes as colorless flat needles which melt sharply and without decomposition at 114.5–115°. It is soluble in ether, acetone, and alcohol and insoluble in petroleum ether, 5% sodium hydroxide, and 5% hydrochloric acid. Solubility in warm water is very slight. Warming for 1 min. in 3 *N* HCl causes the development of a fuchsia color with a slight blue fluorescence. When this solution is made basic, the color changes to light yellow and this material now behaves as an indicator since the colors are reversible to pH changes. Warm 3 *N* NaOH causes extensive decomposition.

Pure xanthatin gives no color with ferric chloride but absorbs bromine readily in carbon tetrachloride solution with no apparent evolution of HBr. No crystalline brominated product could be isolated. It is readily oxidizable by KMnO_4 in alcoholic solution but the product remained amorphous.

The $[\alpha]_D^{30}$ was determined in absolute alcohol solution and found to be -20° .

Composition

A sodium fusion proved the absence of sulfur, nitrogen, and halogen.

A molecular weight determination was carried out utilizing the freezing point depression of *p*-bromotoluene. By this method the molecular weight was 206. The necessity for using extremely small quantities of xanthatin probably decreased the accuracy of the determination.

Microanalysis² of the material melting at 114.5 – 115° gave the following values:

Analysis.— $C_{14}H_{16}O_3$ (232.3). Calculated, C 72.39, H 6.94; found C 72.23, H 7.16.

Xanthatin was found to have a nicely defined absorption spectrum in the ultraviolet ranges as determined on a Beckman model DU spectrophotometer. The absorption is found in two regions as shown in Fig. 2: an intense highly resolved band with the maximum at $275\text{ m}\mu$ ($\epsilon = 2.28 \times 10^4$) and a band of lower intensity which, although incomplete, appears to be approaching a maximum at approximately $213\text{ m}\mu$ ($\epsilon = 7.30 \times 10^3$).

A sample of pure xanthatin in alcohol when treated quantitatively with hydrogen at room temperature and atmospheric pressure in the presence of Adams' platinum catalyst took up the equivalent of 3 moles of hydrogen/mole of xanthatin. This suggests the presence of three unsaturated bonds per molecule, and since the compound is colorless it is probable that the three are unconjugated. The product was a glassy amorphous material which again could not be resolved into crystalline components. During the first stages of the hydrogenation some degradation of the material occurred as shown by the temporary formation of a deep red color identical to that obtained with alcoholic alkali. It is probable that the alkalinity of the Adams' catalyst brought about some hydrolysis of the compound. Hydrogenation destroyed all antibiotic activity.

Fifty mg. of xanthatin was dissolved in 5 ml. of alcohol, 50 ml. of 0.02 *N* NaOH was added, and the solution was made up to 100 ml. After standing for 20 min. at room temperature, an aliquot was titrated with 0.02 *N* HCl. A saponification equivalent of 227 was obtained in

² The microanalysis was carried out by the Clark Microanalytical laboratory, Urbana, Illinois.

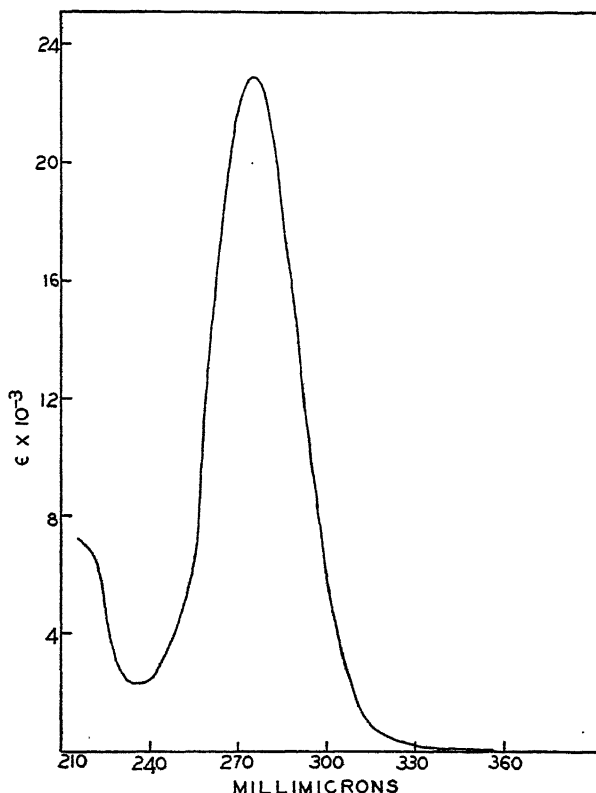


FIG. 2. Ultraviolet light absorption curve for pure xanthatin in absolute alcohol.

this manner. The neutral character of crystalline xanthatin and the ease of hydrolysis suggest a lactone structure.

A further investigation of the chemical structure of xanthatin is now in progress at this laboratory.

Microbiological Aspects

Twenty grams of dried and finely ground plant material was extracted in 50 ml. of absolute ethanol as described under *Methods*. This crude extract showed good activity against *Bacillus subtilis*, *Micrococcus aureus*, *Proteus vulgaris*, *Candida albicans*, and *Trichophyton mentagrophytes*. *Bacillus subtilis* served well as a test organism for the presence

of this impure phytoncide and was used as the cup-test microorganism in titer assays of the various fractions.

In an effort to locate the observed activity in the plant, the stems, leaves, roots, and burs (gathered in August) were assayed separately. The dried and ground plant parts were extracted with ethanol on a 1 g. to 4 ml. basis. Equal weights of each of the four categories were used. Resulting extracts indicated that most of the activity of the mature plant was located in the burs, for the extract of burs was found to contain 1,450 p.p.m. of the pure material, and the extract of dried leaves contained 550 p.p.m. The extract of the stems showed 200 p.p.m., while that of the roots was negligible. Dried mature burs were opened and separated into seeds and fruit walls. These were ground and equal weights extracted with ethanol. Results indicated that all the activity was in the fruit walls. Several different species, including *Xanthium pennsylvanicum* Wallr., *X. leptocarpum* Mill., and Sherff, *X. chinese* Mill.³ as well as others were assayed separately with little or no difference in titer.

The activity of pure xanthatin in ethanol was determined using a selected list of organisms and the resulting spectrum is presented in Table I. The temperature of incubation for each test organism was not always the accepted optimum for the organism under normal conditions of growth, but was the one found best suited to that organism in obtaining an inhibition zone with a clear edge. A great many of the organisms listed in the table are too sensitive to alcohol to permit their use in an agar-streak dilution-type of assay. For comparative purposes, the cup method was found to be the most convenient of any attempted, and the spectrum sufficiently wide to obtain an *in vitro* evaluation of xanthatin.]

Xanthatin has a limited antimicrobial spectrum. With the exception of *Klebsiella pneumoniae*, it possesses negligible activity toward the gram-negative bacteria. As for the gram-positive bacteria, none of the genus *Streptococcus* could be assayed due to alcohol sensitivity, but the various species of *Bacillus* showed xanthatin sensitivity of striking uniformity. The sensitivity of *Micrococcus aureus* was of the same degree as shown in the genus *Bacillus*.

The effect of xanthatin against fungi was noted, with *Ceratostomella*

³ Grateful acknowledgment is expressed to Dr. Earl E. Sherff, Head of the Dept. of Science, Chicago Teachers College, for kindly identifying the plant specimens, and to Dr. Pierre Dansereau, Professor of Botany, University of Montreal, for helpful suggestions in this connection.

TABLE I
Cup Test of Xanthatin in Ethanol^a

| Test organism | Incubation temp. | Xanthatin | |
|---------------------------------------|------------------|-------------|------------|
| | | 1000 p.p.m. | 400 p.p.m. |
| Gram-positive bacteria | C. | | |
| <i>Bacillus subtilis</i> | 37 | 21 | 17 |
| <i>Bacillus mycoides</i> | 28 | 16 | 14 |
| <i>Bacillus megatherium</i> | 28 | 20 | 17 |
| <i>Bacillus cereus</i> | 28 | 20 | 17 |
| <i>Sarcina lutea</i> | 28 | 0 | 0 |
| <i>Micrococcus aureus</i> | 37 | 20 | 16 |
| <i>Mycobacterium phlei</i> | 37 | 15 | 0 |
| <i>Mycobacterium avium</i> | 37 | 15 | 0 |
| <i>Mycobacterium tuberculosis</i> 607 | 37 | 15 | 0 |
| Gram-negative bacteria | | | |
| <i>Escherichia coli</i> | 37 | 0 | 0 |
| <i>Klebsiella pneumoniae</i> | 37 | 23 | 17 |
| <i>Erwinia amylovora</i> | 28 | 0 | 0 |
| <i>Shigella paradysenteriae</i> | 37 | 0 | 0 |
| <i>Alcaligenes fecalis</i> | 37 | 15 | 11 |
| <i>Proteus vulgaris</i> | 37 | 15 | 0 |
| <i>Pseudomonas fluorescens</i> | 37 | 0 | 0 |
| Fungi | | | |
| <i>Aspergillus niger</i> | 28 | 0 | 0 |
| <i>Ceratostomella ulmi</i> | 28 | 35 | 15 |
| <i>Botritis</i> spp. | 28 | 30 | 15 |
| <i>Trichophyton mentagrophytes</i> | 37 | 40 | 30 |
| <i>Candida albicans</i> | 37 | 35 | 28 |
| <i>Penicillium notatum</i> | 28 | 30 | 18 |
| <i>Fusarium oxysporum</i> | 28 | 26 | 17 |
| <i>Stemphylium</i> spp. | 28 | 15 | 10 |
| <i>Alternaria</i> spp. | 28 | 25 | 0 |

^a Zone of inhibition expressed in millimeters. A standard 10-mm. cup was used.

ulmi, *Trichophyton mentagrophytes*, and *Candida albicans* of particular interest.]

SUMMARY

The occurrence, isolation, and characterization of a new antimicrobial agent from the burs and leaves of *Xanthium pennsylvanicum* is discussed. The name, *xanthatin*, was proposed for this compound.

The formula $C_{14}H_{16}O_3$ was determined and the molecule found to contain three unsaturated bonds which are unconjugated and one masked acid group which is probably a lactone.

The compound is inactive against the gram-negative bacteria but effective *in vitro* against gram-positive bacteria and fungi.]

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Sources of the High Energy Content in Energy-Rich Phosphates¹

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INTRODUCTION

Although the idea of high-energy phosphates has been current for 15 years (1), and has proved indispensable for an understanding of energy storage and energy transfer in biological systems, there has not yet appeared any clear explanation as to why certain phosphate compounds contain more energy than do ordinary ester phosphates. Kalckar (2) in his review article attributes to Coryell the statement that the high energy is the result of "opposing resonance," but he does not explain what is meant by the term. Furthermore, as will be brought out below, this explanation is certainly not correct in the case of *enol*-phosphopyruvate, and may not be applicable to the guanidino phosphates.

The opinion is also sometimes expressed that the high energies of some phosphates are due to the substitution of the "secondary" or "tertiary" hydrogen atoms of phosphoric acid. The change in the degree of ionization of the hydrogen atoms on the formation of phosphate esters is certainly a complicating factor, but it is by no means a major reason for the high energy content of the compounds.

This paper will endeavor to explain precisely why some of the phosphates are high-energy compounds, and also to make clear the role played by the change in ionization on hydrolysis, so that the total energy liberated on hydrolysis may be apportioned between the energy change due to the splitting, and the energy of ionization.

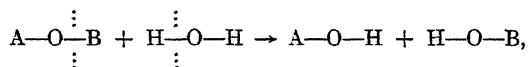
¹ This work was aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

There are four main types of high-energy phosphates, *viz.*:

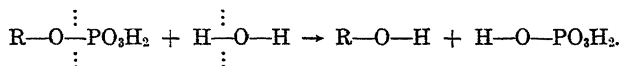
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| (1) Carboxyl phosphate | (1,3-diphosphoglyceric acid, acetyl phosphate). |
| (2) <i>enol</i> -Phosphate | (phosphopyruvic acid, phenyl phosphates). |
| (3) Pyrophosphate | (inorganic pyrophosphate, adenosine diphosphate (ADP), adenosine triphosphate (ATP), cocarboxylase, di- and triphosphopyridine nucleotides, <i>etc.</i>) |
| (4) Amino phosphate | (creatine phosphate, arginine phosphate, aminophosphoric acid). |

Each of these types of compounds will be considered in turn.

It should be pointed out in the beginning that, in the absence of structural peculiarities, one would expect the heat of hydrolysis of any ester (phosphate or not) to be small. For the general reaction may be represented as



and the hydrolysis of a phosphate ester as



The hydrolysis involves the splitting of an O—B bond and an H—O bond (or of an O—P bond and an H—O bond) and then the re-forming of the very same types of bonds. Since the energy of a given type of bond is approximately constant,² there should be little net energy change when a bond is broken, and a like one re-formed.

This argument applies, strictly speaking, only to the heat of the reaction, but since the entropy change on hydrolysis is usually very small, the free energy and the heat of the reaction are nearly the same.

These expectations are usually realized in fact, as is indicated by the classical studies of Berthelot and Pean de St. Giles on the hydrolysis of esters of ordinary organic acids, as well as by the recent work of Kay (3), Ohlmeyer (4), Meyerhof and Green (5), and others, on ordinary phosphate esters. The ΔH values calculated from Berthelot's data are

² This subject is fully discussed in PAULING, *The Nature of the Chemical Bond*, 2nd Ed., pp. 52-58. Cornell University Press.

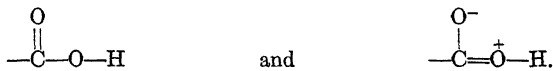
0, and the $\Delta F^{0'}$, about +800 cal./mole; the hydrolysis constants of ordinary phosphate esters correspond to $\Delta F^{0'}$ values of about -400 cal./mole.

These $\Delta F^{0'}$ values are obtained if the standard states of all substances, including water, are taken as 1 *M*. The values (ΔF^0) reported in the literature usually define the standard state of water as pure water; this is often convenient, but to single out one substance in the reaction for special treatment would obscure the argument presented here. The values of ΔF^0 and $\Delta F^{0'}$ differ by $RT \ln 55 = 2200$ cal.

I. CARBOXYL PHOSPHATE

The free energy of hydrolysis of carboxyl phosphate is -16,000 cal./mole (6). In other words, the carboxyl phosphates are 16,000 cal. less stable than the hydrolysis products. The explanation in this case, is indeed "opposing resonance."

The carboxyl group resonates between the two structures



The resonance energy is 28,000 cal./mole for carboxylic acids, which means that the compound is 28,000 cal. more stable than it would be if this resonance did not occur.

The phosphate ion HPO_4^{--} , on the other hand, resonates among the structures (Fig. 1), with various arrangements of the bonds and charges. The structures are arranged according to the number of double bonds to the central P atom, and then according to the number of ionized oxygen atoms. The numbers in parentheses under the formulas indicate the number of resonating structures belonging to each type. There are, for example, three structures of type *B*, since the double bond can resonate among the three oxygen atoms not bound to the hydrogen; when the double bond goes to the hydrogen-bonded oxygen, the structure is designated as a new type, *C*.

Of these 50 resonating forms, the 19 of types *D*, *E*, *G*, *K*, *O*, *P*, and *Q* are very unstable, because they have charges of the same sign on adjacent atoms; this gives rise to strong coulomb repulsions, which require large amounts of energy to overcome. Being unstable, these forms make practically no contribution to the total structure of the molecule.³ The

³ For a complete discussion of the "adjacent charge rule," cf. PAULING, *loc. cit.*, pp. 199, 209-10.

two forms *F* and *H* are also improbable, because of the multiple charges on a single atom. There remain, then, 29 forms (of types *A*, *B*, *C*, *I*, *J*, *L*, *M*, *N*, and *R*) which contribute to the structure of the phosphate ion, and increase its stability.⁴

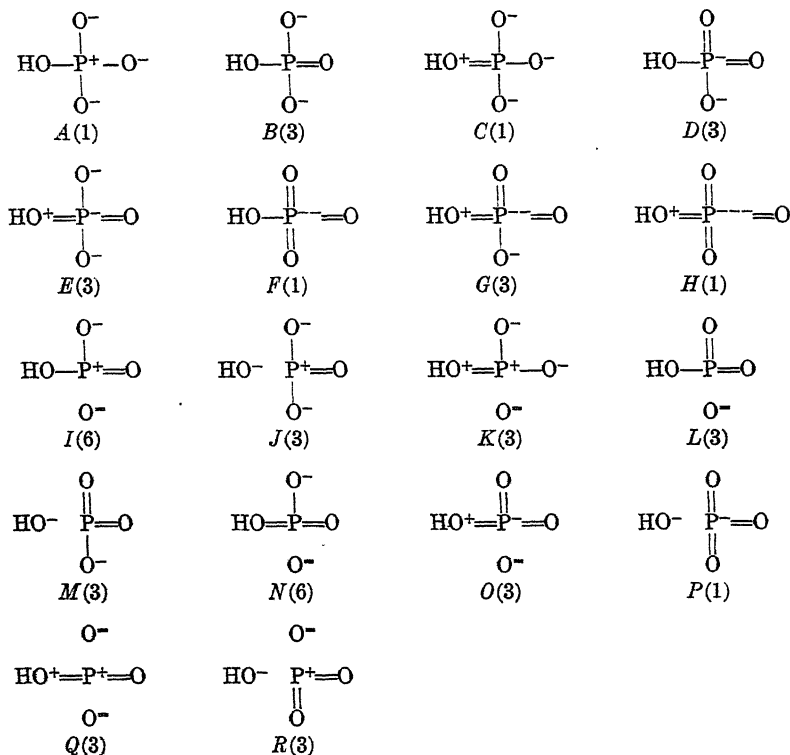


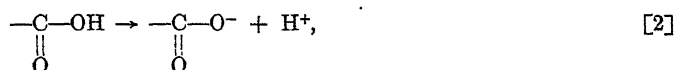
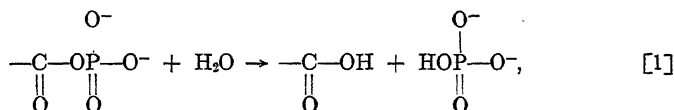
FIG. 1.

Carboxyl phosphate is formed by splitting out a molecule of water between the carboxyl group and the phosphate ion, and if the resonance of both groups were complete, there should be 58 (2×29) resonating forms of carboxyl phosphate, since either of the two resonating forms

⁴The same considerations apply in essence, though not in detail, to the other ionization states of phosphate. The ion H_2PO_4^- has (Fig. 2) 22 unstable forms, plus three improbable ones, leaving 25 contributing structures. The triply-ionized form has 14 unstable, 1 improbable, and 35 contributing structures; unionized H_2PO_4 has 23 unstable, 4 improbable, and 23 contributing structures.

of the carboxyl group should be able to combine with any of the 29 forms of the phosphate ion. This is not the case, however. The first resonating form of the carboxyl group, is indeed compatible with all the resonating structures of the phosphate ion, for here the H atom of the phosphate has merely been replaced by an acyl group, but in the second resonance form of the carboxyl, the connecting oxygen is doubly bonded to the carbon (as it cannot be to hydrogen), and is therefore one charge unit more positive than in the phosphate ion. The result is that the seven structures of types *A* and *I* now become impossible, and the seven structures of types *C* and *N* become improbable, while the single structure of type *P* becomes possible. There is thus a net loss of 13 structures because of the incompatibility of complete resonance in both the phosphate and the carboxyl groups. This phenomenon, which has been termed "opposing resonance," is the source of the thermodynamic instability of carboxyl phosphates.

One further point may be considered at this time. If a carboxyl phosphate is hydrolyzed at physiological pH, the total reaction is hydrolysis, followed by ionization of the carboxylic acid formed, *viz.*:



where $-\text{COOPO}_3^{--}$, HOPO_3^{--} , and $-\text{COO}^-$ are the ionization forms predominating at physiological pH.

A portion of the free-energy change observed is due to the second reaction; this contribution may be calculated from the well-known equation

$$\Delta F_2 = -RT \ln K_2 + RT \ln \frac{(\text{H}^+) (\text{COO}^-)}{(-\text{COOH})}$$

and since we are concerned with the standard free-energy change, the concentrations of the acid and the ion formed from it are taken as unity, so that $\Delta F_2 = RT \ln \frac{(\text{H}^+)}{K_2}$, which depends both on the pH of the medium, and on the ionization constant of the acid. For the average carboxylic acid ($K = 2 \times 10^{-5}$) and at pH 7, we have $\Delta F_2 = -3200$ cal. Hence,

only about 13,000 cal. of the free-energy decrease on hydrolysis is due to the breakup of the high-energy compound, and the remaining 3000 cal. are attributable to the ionization at the pH at which the reaction is carried out.⁵

It is apparent that the observed *heat* of the reaction is also the sum of the heats of hydrolysis and of ionization. Meyerhof and Schulz (7) who measured the heats of acid hydrolysis of guanidino phosphates, have corrected their figures for the ionization heats, and obtained the values for the heats of hydrolysis of the ionization forms existing at the neutral point, to give the split products in the ionization forms in which they exist at the neutral point.

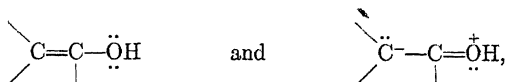
The above considerations apply to the ΔF and ΔH of splitting of the esters in the pure state, but since the pH of the medium is usually maintained constant by means of a buffer, and the ionization of the split products is accompanied by a change in ionization of the buffer, it is appropriate to inquire what effect this has upon the ΔF and ΔH actually observed. In the case of ΔF , the answer is none, because the ionization of the buffer takes place at its equilibrium point, where $\Delta F = 0$. The heat of ionization of the buffer is, however, not generally 0, so that the observed ΔH of splitting in a buffer solution is equal to the ΔH of the actual splitting, plus the difference between the heats of ionization of the split products and the buffer. This depends, of course, upon the nature of the buffer, as well as upon the pH, which determines the extent of ionization of the unsplit ester and the split products.⁶ It may be pointed out, however, that heats of ionization are usually small, and their differences are even smaller.

⁵ Since the second ionization constant of the phosphate increases when the phosphate is esterified, allowance should also be made for the free energy of recombination of hydrogen and phosphate ions which follows the hydrolysis. The computation is exactly like that given for the ionization of the carboxyl group, but the magnitude of the effect is much less (80 cal.), because $\frac{2}{3}$ of the 2nd group of the phosphate remains dissociated (at pH 7) even in the unesterified form, and also because the recombination takes place at a pH very near the second pK of phosphoric acid.

⁶ Meyerhof and Lohmann (8) called attention to this fact in connection with their measurement of the heat of deamination of adenylic acid. The heat varied, depending upon the amount of protein in the enzyme solution, because when the protein decreased, a larger fraction of the ammonia released was neutralized by the phosphate group instead of by the enzyme protein.

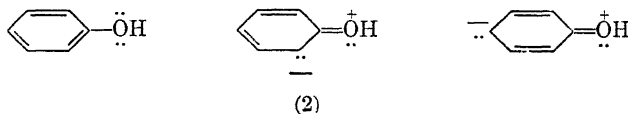
II. ENOL-PHOSPHATE

The heat of hydrolysis of *enol*-phosphopyruvate has been found by Meyerhof and Schulz (7) to be 8500 cal. Here, however, the concept of opposing resonance does not provide an adequate explanation for the high energy of the compound. It is true that the enol group does resonate between the structures



which are analogous to those of the carboxyl group, but the resonance energy is too small to account for the observed heat of hydrolysis of *enol*-phosphate.

It is unfortunate that the resonance energy of the enol group is not known exactly, but the phenol molecule, which resonates among the four structures

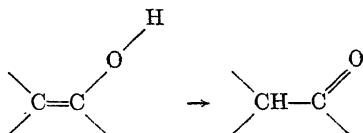


has only 7 kcal. of resonance energy in excess of that in the benzene ring. Since phenol has four structures, compared with the two of ordinary enols, and contains the benzene ring, which is conducive to resonance, the resonance energy of the enols must be a mere fraction of that of phenol, or not more than 2 or 3 kcal. at most. Hence, it would be possible to give up the entire resonance energy of the enol group without incurring a loss in stability equal to that of the *enol*-phosphates.

The energy of hydrolysis of *enol*-phosphate is explained by the fact that the enol form is unstable relative to the carbonyl form of the molecule. Hydrolyzing the phosphate does not in itself yield much energy, but it does make it possible for the free enol to revert to the more stable carbonyl form, with the evolution of a large additional amount of energy.

The exact heat of the enol-keto transformation has not been measured, but if one assumes that the energy of a given type of bond is constant (cf. above) and uses the table of bond energies given by

Pauling,⁷ one finds that the transformation



involves forming the bonds

| | |
|---------------|----------|
| C—C, yielding | 59 kcal. |
| C=O " | 152 |
| C—H " | 87 |
| | <hr/> |
| | 298 |

and breaking the bonds

| | |
|-------------|-----------|
| C=C, losing | 100 kcal. |
| C—O " | 70 |
| O—H | 110 |
| | <hr/> |
| | 280 |

Accordingly, the enol form would be 18 kcal. less stable than the keto form. A more exact calculation, based on the table of Andersen, Beyer, and Watson (9), which takes some account of the structural peculiarities of the compounds, gives a value of 9 kcal.; the authors state that, in general, the error in the heat of formation of a compound as calculated from this table is less than 4 kcal. (These calculations are for molecules in the gaseous state; any difference in the heats of solution of the enol and keto forms would of course have to be added.)

It is clear in any case that *enol*-pyruvate is much less stable than the keto form, and that the energy of transformation accounts for most of the heat of hydrolysis of *enol*-phosphopyruvate.⁸

⁷ *Loc. cit.*, pp. 53, 131.

⁸ Additional evidence as to the energy difference between the enol and keto forms may be obtained from the fact that pyruvic acid does not occur in the enol form to any appreciable extent. (Henri and Fromageot (11) found spectroscopic evidence for the presence of traces of the enol form, but were unable to determine the amount present.) Acetoacetate contains about 8% of the enol form, indicating that the enol is about 2 kcal. less stable than the keto form ($\Delta F = RT \ln K$) but here the enol form is stabilized relative to the keto by the presence of conjugated double bonds, with a resonance energy of about 5 kcal., so that in the absence of this factor the total instability of the enol form would be about 7 kcal.

The free energy of hydrolysis of phosphopyruvate has been found experimentally (10) to be $-16,000$ cal.; *i.e.*, about 7000 cal. more than the heat. It is impossible to assign any part of this difference to ionization of the acid group liberated (as in the case of the carboxyl phosphates discussed above) because the enol group does not ionize at physiological pH. It appears that there must be a large entropy term involved, but it is not clear why the entropy should increase so markedly, either on hydrolysis or on conversion of the enol to the keto form.

In this connection it may be appropriate to discuss phenyl phosphate, and the nitrophenyl phosphates, which are physiologically active, and which have been found by Axelrod (12) to transphosphorylate with a number of alcohols.

Resonance among the four phenolic structures given above stabilizes the phenol molecule by 7 kcal. Since the last three structures have a double-bonded oxygen atom, it is apparent that formation of phenyl phosphate will result in opposing resonance, and render the compound unstable by something less than 7 kcal. It may be predicted, therefore, that the heat of hydrolysis of phenyl phosphate will be about 5 kcal.; this would definitely place the compound in the high-energy class.

Turning to the *o*- and *p*-nitrophenols, we observe that, in addition to the usual resonating structures of phenol and nitrobenzene, there is also a structure



which is about as important as the other ionic structures of phenol. The extra stability contributed by this structure may be estimated⁹ as 2 kcal.; since this structure, too, is incompatible with complete resonance in the phosphate group, the heat of hydrolysis of *o*- or *p*-nitrophenyl phosphate would be about 7 kcal.

⁹ It is usually possible to determine resonance energies exactly from observed heats of formation. In this case, however, although the heats of formation of the nitrophenols have been measured, they are not sufficiently accurate to reveal resonance energies of this order of magnitude.

The 2,4 and 2,6-dinitrophenols, with two *o*- or *p*-nitro groups have twice as many extra structures, and the heats of hydrolysis of their phosphates would probably be about 8 kcal.

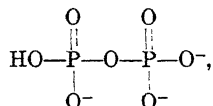
In *m*-nitrophenol, the extra structure does not occur; there is even some incompatibility between the phenol- and nitrobenzene resonance. This compound has, therefore, even less resonance energy than phenol, and the heat of hydrolysis of its phosphate would be expected to be less than that of phenyl phosphate, though probably not much less.

Phenolphthalein phosphate is like phenyl phosphate.

III. PYROPHOSPHATES

All compounds containing the pyrophosphate bond are high-energy compounds, because they exhibit opposing resonance.

The form of inorganic pyrophosphate which predominates at physiological pH is



which hydrolyzes to H_2PO_4^- , and HPO_4^{--} . The resonating forms of the latter have already been discussed; those of the former are given in Fig. 2. Only the 25 forms belonging to types *a*, *b*, *c*, *d*, *j*, *l*, *n*, *o*, *p*, *q*, and *v* are important, for the reasons discussed above.

The pyrophosphate ion must be thought of as a combination of the two ions H_2PO_4^- , and HPO_4^{--} , with the central oxygen serving both as the hydroxyl oxygen of HPO_4^{--} and as one of the hydroxyl oxygens of H_2PO_4^- . Complete resonance in both halves of the ion is impossible, for when the central oxygen is doubly bonded to the phosphorus atom on the left (which gives H_2PO_4^-) it becomes one charge unit more positive, and we have seen that this results in a net loss of 13 resonating structures in the right-hand half of the molecule (which hydrolyzes to HPO_4^{--}). There are seven structures¹⁰ which doubly-bond one particular hydroxyl oxygen to the phosphorus atom in H_2PO_4^- , entailing a loss of 91 (7×13) structures in pyrophosphate. On the other hand, the six structures which ionize the oxygen¹¹ make it one unit more negative, and render possible the six structures of types *K* and *Q* in the

¹⁰ One type *c*, 2 *n*, 2 *o*, 2 *q*.

¹¹ Two type *l*, 1 *p*, 2 *q*, 1 *v*.

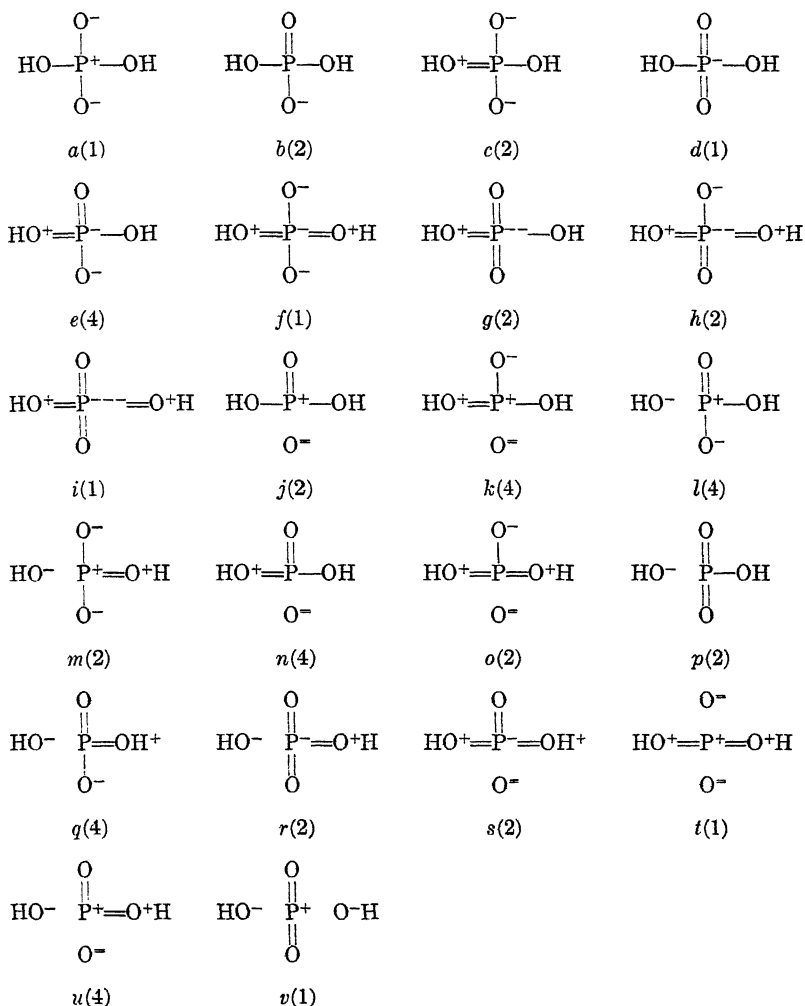


FIG. 2.

right-hand half of the molecule. The resultant gain of 36 structures, when subtracted from the loss of 91, gives an over-all loss of 55 structures, and a considerable decrease in stability.

The actual heat of hydrolysis of pyrophosphate calculated from the heats of formation of $\text{HP}_2\text{O}_7^{--} + \text{H}_2\text{O}$ vs. $\text{HPO}_4^{--} + \text{H}_2\text{PO}_4^-$ (13)

is 6.2 kcal. An experimental value of 4.3 kcal. has been obtained in acid solution (14); this value, corrected for ionization to physiological pH, would probably be in very good agreement with the calculated figure. The heats of hydrolysis of the pyrophosphate bonds in ATP and ADP are, of course, 12 kcal. per bond (8).

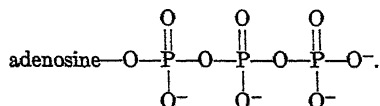
It may be noted that there is very little free energy of neutralization when pyrophosphate is hydrolyzed at pH 7. Only about 0.5 mole of acid are formed/mole of pyrophosphate split, and the free energy/mole is small, because the pH is close to pK.

Cocarcboxylase is simply pyrophosphate with the hydrogen replaced by a thiamine group, so that the preceding discussion applies equally well to this compound. The same holds true for *adenosine diphosphate*, where the hydrogen has been replaced by adenosine.

Diphosphopyridine nucleotide (DPN) also contains a pyrophosphate linkage, which would give two substituted H_2PO_4^- ions if it hydrolyzed. Complete resonance in both halves of the molecule is again impossible; if the central oxygen atom became one charge unit more positive by double-bonding to one phosphorus atom, there would be a net loss of 11 structures¹² of the other phosphate group. We have seen that there are 7 such double-bonded structures, giving a total loss of 77 structures. Furthermore, if the oxygen atom becomes one charge unit more negative, by ionization from one phosphorus atom, there is a net loss of two structures¹³ of the other phosphate group. Since there are 6 such ionic structures, there results an additional loss of 12 structures, giving a grand total of 89 resonating structures which are lost. (The difference between 89 and the 55 calculated above for inorganic pyrophosphate, is due, of course, to the difference in the ionization states of the phosphates formed by hydrolysis.)

Flavine adenine dinucleotide is exactly similar to DPN.

Adenosine triphosphate at physiological pH exists chiefly in the form



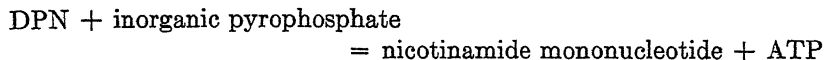
Opposing resonance between the central and the left-hand phosphate groups results in a loss of 89 structures (as in the DPN molecule dis-

¹² One type *a*, 1 *c*, 2 *j*, 2 *l*, 2 *n*, 2 *o*, and 2 *q* become impossible or improbable, while 1 *r*, formerly impossible, becomes possible.

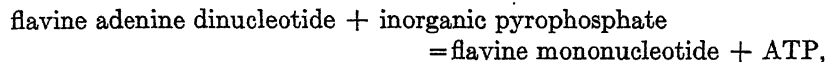
¹³ One type *d*, 2 *l*, 1 *p*, 2 *q*, 1 *v* are lost, while 2 *k*, 1 *m*, 2 *u* become possible.

cussed above), and opposing resonance between the central and right-hand phosphate groups results in a further loss of 55 structures (as in inorganic pyrophosphate).

Kornberg (15) has recently called attention to the fact that the equilibrium constant of the reaction

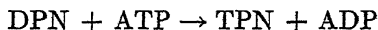


is of the order of magnitude of one which corresponds to a free-energy change of zero, confirming the idea that all the pyrophosphate linkages on each side of the equation are about equally unstable. This may be taken as experimental evidence for the ideas discussed in this section; it is to be expected, of course, that the thus-far unmeasured equilibrium constant of the reaction



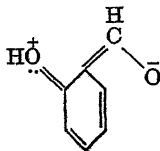
which is very similar to the reaction involving DPN, will also be of the same order of magnitude.

Kornberg (16) has found that the third phosphate of triphosphopyridine nucleotide (TPN) is probably linked to the second hydroxyl of the ribose group. This means that it is a low-energy phosphate group, and that the reaction

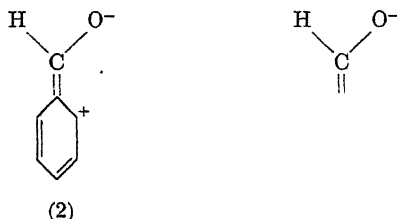


discovered by Kornberg (17) involves the loss of one high-energy phosphate. Accordingly, it must take place with a decrease in free energy of approximately 12,000 cal.; this means that it would run practically to completion from left to right.

The structure of pyridoxal phosphate is in some doubt. It may be pointed out here that if the phosphate were esterified with the aliphatic hydroxyl group, it would be a low-energy phosphate. If the phenolic hydroxyl is phosphorylated, a compound of moderately high energy will result, because of the resonating structures of phenol which doubly-bond the oxygen to the ring; in the case of the pyridoxal, there is also a structure



so that the instability of the phosphate would be approximately the same as that of *o*- or *p*-nitrophenol phosphate. Finally, if the phosphate were attached to the aldehyde group, forming a compound analagous to a hemiacetal, a really high-energy compound would result. The reason for the great instability is similar to the reason for the instability of *enol*-phosphopyruvate. When the compound is hydrolyzed, it would become possible for the pyridoxal to revert to the normal aldehyde structure; this involves the formation of a C=O bond, and the breaking of two C—O bonds (the O—H bonds cancel out). According to Pauling's table (*vide supra*) the process would involve a net evolution of 9 kcal. in the case of an ordinary aldehyde, but in the case of a benzaldehyde, resonance to the structures



provides an extra 4 kcal. of stability for the aldehyde but not for the acetal. The structure given above for pyridoxal would add approximately another kilocalorie, giving a total of about 14 kcal. for the heat of hydrolysis of the phosphate with the hemiacetal structure.

IV. GUANIDINO PHOSPHATES

The guanidino phosphates, and even aminophosphoric acid, are high-energy compounds, but it must be pointed out at once that the hydrolysis of these compounds involves the rupture of N—P and H—O bonds, followed by the formation of N—H and O—P bonds. There is no *a priori* reason to expect that such a reaction would be thermoneutral, and there is no experimental value for the energy of the N—P bond. To a first approximation, the heats and free energies of hydrolysis are due mainly to the neutralization of the bases formed by the hydrolysis.

The heat of hydrolysis of aminophosphoric acid is 14 kcal. (8). This is due in part to the heat of neutralization of the ammonia liberated. It is not apparent, however, why the heats of hydrolysis of creatine phosphate (11 kcal.) and arginine phosphate (8 kcal.) should be smaller. Both creatine and arginine are strong bases, whose heats of neutraliza-

tion are no smaller than that of ammonia. Furthermore, the phenomenon of opposing resonance can occur in the guanidino phosphates, since guanidine resonates as follows:



The structure with the double bond to the phosphorylated nitrogen atom is obviously incompatible with many of the resonating phosphate structures. Hence, one would expect the guanidino phosphates to be much more unstable than aminophosphoric acid. There is evidence (18), however, that the aminophosphoric acid is actually a polymetaphosphoric acid; if this is true, the situation would probably be as follows: the change in bonds (from $\text{N}-\text{P} + \text{H}-\text{O}$ to $\text{P}-\text{O} + \text{N}-\text{H}$) absorbs considerable energy, but this is more than counterbalanced by the partial neutralization of the base formed, plus the hydration of metaphosphate (in aminophosphoric acid) and the elimination of opposing resonance (in guanidino phosphates).

The relative heats of hydrolysis of creatine phosphate and arginine phosphate are about what one might expect. Substitution of one of the amino groups of guanidine tends to prevent the double bond from swinging to the nitrogen of that group; hence, in creatine, where one amino group is doubly substituted, the structure with the double bond to that nitrogen is of little importance, and phosphorylation of the other group practically eliminates all of the resonance remaining in the molecule, resulting in great thermodynamic instability. With arginine, on the other hand, there is still an appreciable contribution of the structure with a double bond to the alkyl-substituted nitrogen, and phosphorylation of the second amino group cannot remove quite all of the resonance.

Much of the free energy of hydrolysis of the guanidino phosphates is also due to the neutralization of the base liberated:

$$-\Delta F = RT \ln \frac{(K_b)(\text{H}^+)}{K_w}$$

(cf. the discussion of carboxyl phosphate). This means that the phosphate tends to split with increasing hydrogen-ion concentration and is

in accord with Lehmann's finding (19) that the equilibrium of the reaction



shifts to the right with increasing acidity.

ACKNOWLEDGMENT

I wish to thank Dr. Otto Meyerhof for his kind interest in this work.

SUMMARY

The reasons for the instability of the following high-energy phosphates have been discussed: carboxyl phosphates, *enol*-phosphates, phenyl (and substituted-phenyl) phosphates, inorganic pyrophosphate, adenosine diphosphate, adenosine triphosphate, di- and triphosphopyridine nucleotides, flavine adenine dinucleotide, and amino phosphates. The structures of triphosphopyridine nucleotide and pyridoxal phosphate were discussed.

The role of the ionization of both the phosphate compounds and the buffer medium was considered with respect to its effect on the heat and the free energy of hydrolysis.

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Microanalytical Determination of the Rate of Tobacco Mosaic Virus Synthesis in Tobacco Leaf Tissue¹

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INTRODUCTION

Plants infected with a well-characterized virus, such as tobacco mosaic, offer numerous possibilities for investigation of the processes related to reduplication of nucleoproteins, and the manner in which such substances exercise their profound determinative effects.

An essential requirement for the development of such studies is an accurate method for determining the amount of virus protein present in localized tissues of the host. Although sensitive and dependable procedures for estimating relative virus contents of plant sap by local lesion counts have been worked out (11), these techniques measure the number of infective units rather than the amount of virus protein present. Since virus particles may form aggregates of a size which varies with the ionic strength, pH, and other properties of the extract (14), such counts cannot be related to the absolute virus content of unknown samples.

We have therefore been interested in developing a micro method for the determination of the tobacco mosaic virus content of small masses of infected tissue, with a view to applying this procedure to an analysis of the events precipitated by the entry of the virus into host cells. The present paper is a description of such a method and its application to the problem of estimating the rate of virus synthesis in tissue from single tobacco leaves.

¹ This work was supported in part by a grant from the American Cancer Society, recommended by the Committee on Growth.

EXPERIMENTAL AND RESULTS

I. Quantitative Determination of Microgram Quantities of TMV by Isoelectric Precipitation

For this work, standard preparations of purified tobacco mosaic virus (TMV) were made from leaves of plants (*Nicotiana tabacum*, var. *White Burley*) systemically infected with the Johnson strain of tobacco mosaic virus by inoculation at least 2 months previous to the time of isolation. The virus protein was isolated from the fluid obtained by homogenizing tissue (from leaves with characteristic symptoms) in twice its volume of pH 7.0 phosphate buffer. Normal proteins are first removed by bringing the material to pH 4.2 and discarding the precipitate. When the supernatant is brought to pH 3.4 the virus precipitates out as the typical microneedles first described by Stanley (12) and may then be purified by dissolving at pH 7.0 and reprecipitating at pH 3.4. Figure 1 shows that two such precipitations yield a product with an ultraviolet absorption spectrum characteristic of TMV [see Bawden and Pirie (2) and Lavin and

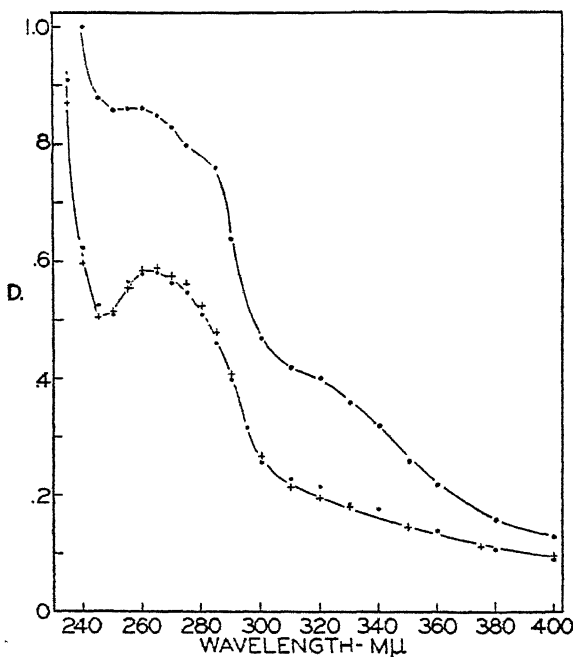


FIG. 1. The absorption spectrum of three successive precipitations of protein of TMV-infected leaves from pH 3.4 buffer. Upper curve, first precipitation; lower curve, second precipitation (dots), and third precipitation (plus signs). All preparations were redissolved in pH 7.0 phosphate buffer at equivalent dilutions.

TABLE I
Characteristics of Purified Virus Preparations

| Preparation | Isolation method: pH of second centrifugation | N | P | Extinction at 260 m μ per mg./ml. | Folin value: extinction at 750 m μ per mg./ml. | $\frac{E_{260 \text{ m}\mu}}{E_{280 \text{ m}\mu}}$ | Infectivity: lesions/sq. cm. leaf/10 μ g./ml. TMV |
|----------------|---|-----------|-----------|---------------------------------------|--|---|---|
| A ^a | 4.2 | % 16.0 | % 0.57 | 3.30 | 16.7 | 1.18 | — |
| B ^a | 4.2 | 16.5 | 0.55 | 2.96 | 18.8 | 1.20 | 1.2 |
| C ^a | 4.2 | 16.3 | 0.51 | 2.88 | 17.7 | 1.18 | 2.7 |
| D ^b | 4.5 | — | — | 2.85 | 17.6 | 1.19 | 2.0 |
| E ^a | 4.7 | 16.6 | 0.55 | 2.93 | 17.6 | 1.18 | 1.7 |
| F ^b | 4.7 | — | — | 2.95 | 17.3 | 1.20 | 0.8 |

^a Dry weight determined.

^b Dry weight calculated from N-determined $\times 6$.

^c On leaves of *N. tabacum-glutinosa* hybrid.

Stanley (9)]. That this material has the properties previously reported for purified TMV can be seen from determinations of nitrogen (micro-Kjeldahl), phosphorus (SubbaRow-Fiske), specific extinction at 260 m μ , and infectivity. The results obtained from a series of preparations purified in the above manner are given in Table I; the properties agree closely with those previously reported by Bawden (1) and Stanley and Loring (13).

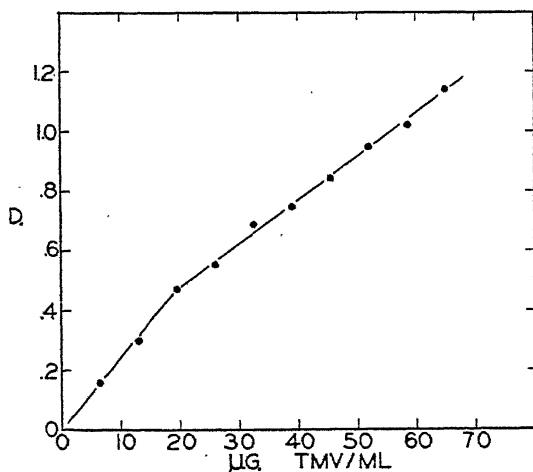


FIG. 2. Folin calibration curve for TMV protein. The TMV content is based on dry-weight determinations of the purified sample used. The optical density is for 750 m μ .

A modification of the Folin reaction by Lowry *et al.* (10), based on a procedure described by Kabat and Mayer (8) was found to give accurate determinations of 10–100 $\mu\text{g.}$ of TMV. The procedure is carried out in small graduated centrifuge tubes (7 mm. in diameter). To the virus protein (in a solvent volume of 0.05 ml.) is added 0.85 ml. of a modified alkaline reagent (4% Na_2CO_3 , 20 parts; 2% sodium tartrate, 1 part; 1% CuSO_4 , 1 Part). The mixture is allowed to stand at room temperature for 30 min. at which time 0.1 ml. of a mixture of 1 part Folin phenol reagent and 2 parts distilled water is added. This is mixed instantaneously with a small magnetic stirrer. The color is read at its absorption maximum (750 $\text{m}\mu$) after 30 min., using microcells and a Beckman spectrophotometer. For other total volumes, the amounts cited above are altered appropriately.

The reaction was calibrated by determining the extinctions yielded by 5–65 $\mu\text{g./ml.}$ of purified TMV (determined by dry weight). The results are shown in Fig. 2. While the reaction does not follow Beer's law over the entire range studied, the curve is reproducible and can therefore be used to evaluate the virus content of unknown samples. In practice the volume is chosen to bring the extinction into the range shown in Fig. 2. Determinations are accurate to within 3–4 $\mu\text{g.}$ of TMV.

Using the above procedure, the completeness of precipitation of purified TMV at various pH's was determined. One-ml. aliquots, each containing 40 $\mu\text{g.}$ of purified TMV in 0.1 *M* phthalate buffer, were centrifuged at 1700 relative centrifugal force

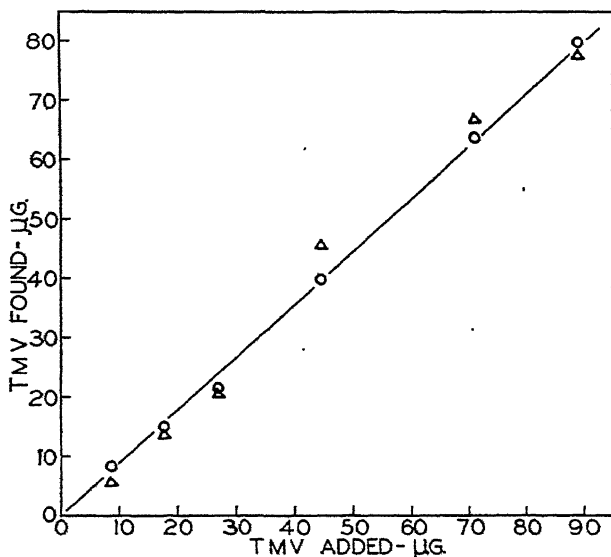


FIG. 3. Recovery of TMV protein by precipitation at pH 3.4. TMV was added as purified material and the precipitate determined by the Folin method. Circles represent precipitates determined without washing; triangles represent equivalent precipitates determined after recentrifuging from a wash with pH 3.4 buffer.

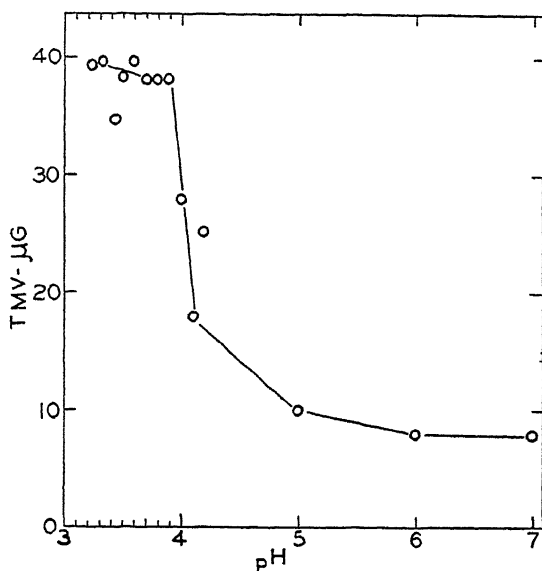


Fig. 4. Recovery of 40 μ g. of purified TMV protein by centrifugation at various pH's.

(R.C.F.) for 30 min. after remaining at the specified pH overnight at 10°C. The amount of virus recovered from the precipitate at various pH's (determined by the Folin method) is shown in Fig. 4, which indicates that recovery is essentially complete when the pH is less than 3.8. This was confirmed by recovering 8–90 μ g. of purified TMV from 1 ml. of phthalate buffer by centrifugation at pH 3.4. As can be seen from Fig. 3, these amounts of TMV can be recovered with a yield of about 90%.

II. The Microanalytical Procedure

Hills and McKinney (7) have shown that the virus content of 30-g. lots of infected tobacco leaves may be estimated by isoelectric precipitation of the virus protein after precipitation of non-virus protein at pH 4.2. However, when their method was applied to healthy leaf tissue, the amount of protein isolated was about 10% of the amount of virus yielded by a corresponding mass of infected tissue. Preliminary studies of their method by us also indicated that a significant amount of virus was lost with the non-virus protein discarded after centrifugation at pH 4.2; this is in agreement with the data shown in Fig. 4. Hence to provide a basis for developing an accurate micro-analytical method, the following procedure was employed to determine the optimum pH for the removal of non-virus protein from leaf homogenates.

The starting material was of the order of 50 mg. wet weight of leaf-blade tissue. This was homogenized at 0°C. in a small glass homogenizer in 0.5 ml. of 0.1 *M* phthalate buffer (pH 7.0, and 0.5 *M* with respect to sucrose). This concentration of

sucrose prevented rupture of the chloroplasts, and analysis of the extracts thereby yields the non-chloroplast virus content of the leaf.

After standing overnight, the homogenate was centrifuged, the supernatant brought to the particular pH to be tested, and again stored overnight. It was then centrifuged and the precipitate discarded. The supernatant was adjusted to pH 3.4, stored overnight, and again centrifuged. The precipitate was redissolved in 1.0 ml. of pH 7.0 phthalate buffer (without sucrose), centrifuged, and the supernatant readjusted to pH 3.4. After standing overnight, the precipitate was centrifuged out, washed with pH 3.4 phthalate buffer, and again centrifuged. The supernatant was drawn off to 0.05 ml. and the TMV content of the precipitate determined by the Folin procedure outlined above. In most cases, the final precipitate obtained from infected tissue consisted of characteristic microneedles.

All pH adjustments were done in 5-ml. beakers using a small glass electrode. The material was centrifuged in small calibrated tubes, 7×200 mm., at 13-1700 R.C.F. for 30 min. using an angle-head. Transfers from beakers to centrifuge tubes were done with washing. Transfers from centrifuge tubes to beakers were made by removing a specific determined fraction of the supernatant (90 or 95%).

A series of 1.2-ml. aliquots of a homogenate of leaf blade from a systemically-infected tobacco plant were prepared, each sample representing an area of 302 sq. mm., or 50 mg. (wet weight) of leaf tissue. The samples were then treated as outlined above, each being brought to a different pH after centrifugation at pH 7.0. With the exception of

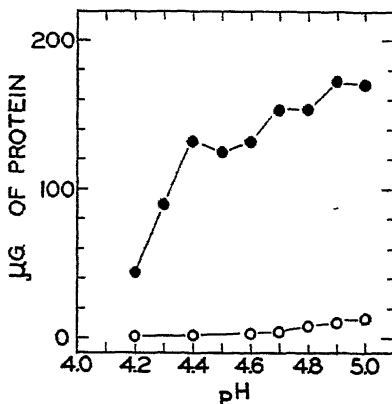


FIG. 5. Effect of various pH's at second centrifugation on amount of protein recovered from aliquots of homogenates of infected (closed circles) and healthy (open circles) leaf tissue. Each point represents analysis of an aliquot containing 50 mg. wet weight of tissue. In all cases the analytical procedure was that shown in the scheme of Fig. 6, except that the pH at which the second centrifugation was carried out was varied.

this step all samples were given identical treatment. A corresponding set of aliquots of a homogenate prepared from healthy leaf tissue was run through a parallel series of determinations. The results of analyses of the final precipitates obtained from infected and healthy tissues are shown in Fig. 5. It is apparent that the method serves to isolate virus protein from homogenate of infected leaves.

It will be noted from Fig. 5 that the maximum yield of protein from infected tissue (at pH 5.0) is 170 $\mu\text{g.}/302$ sq. mm. of leaf. Since healthy tissue yields protein equivalent to 10 $\mu\text{g.}$ of TMV when treated at this pH, the maximum yield of protein characteristic of infected tissue is 160 $\mu\text{g.}$ With treatment at pH 4.7, the corresponding figures are 156 $\mu\text{g.}$, 2 $\mu\text{g.}$, and 154 $\mu\text{g.}$ Hence, if the procedure involves a second centrifugation at 4.7, it is apparent that the expected return of virus protein from infected tissue will be at least 93% of the maximum; while healthy tissue similarly analyzed will give protein values of essentially zero magnitude.

As a confirmation of the identity of the protein precipitated at pH 3.4 after a centrifugation at pH 4.7, a larger quantity of material was isolated from infected leaf tissue according to this procedure and characterized as to N and P content, ultraviolet absorption spectrum, and infectivity. Comparison of this product with the material described in Section I (in which the second centrifugation was at pH 4.2) shows that both types of preparation correspond in these properties to the known characteristics of tobacco mosaic virus. (See Table I.)

On the basis of these results the analytical scheme outlined in Fig. 6 may be adopted as a means of achieving reliable microanalyses of 10–50-mg. quantities of tobacco leaf tissue. The quantitative validity of the entire scheme was tested by the procedures described below. All results hereafter described were obtained by the method shown in Fig. 6.

Homogenates of infected and healthy leaf tissue were prepared. From these, artificially-mixed samples containing varying percentages of infected material were made. Each of these was 1.0 ml. in volume and represented 50 mg. of tissue. Each sample was analyzed by the method described above. The results, shown in Fig. 7, indicate that the amount of virus yielded is proportional to the fraction of infected homogenate present. The standard deviation, calculated from the variation of the virus found from the amount expected (on the basis of the virus content of the undiluted virus-containing homogenates themselves), is $\pm 5\%$.

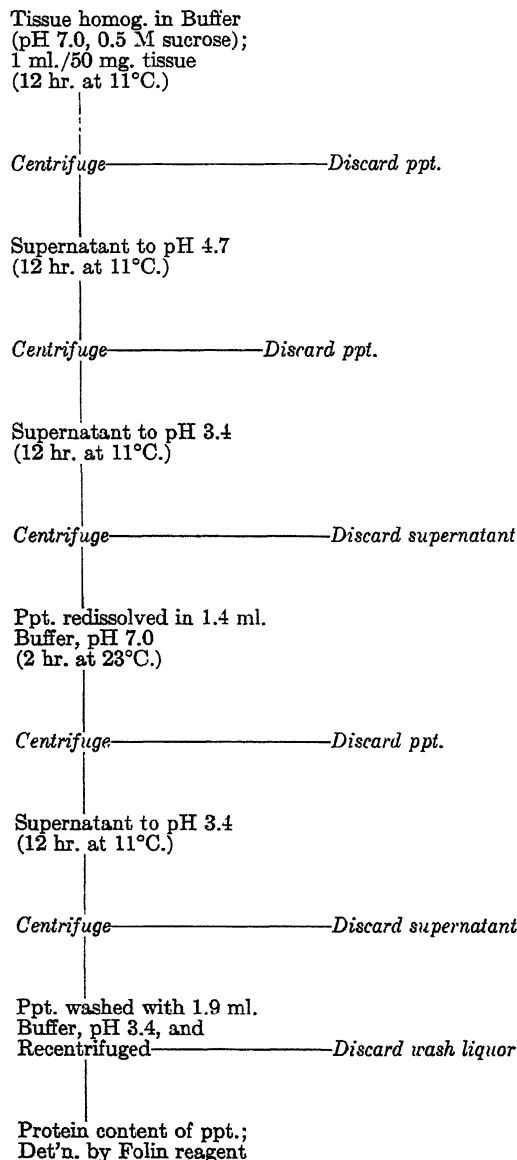


FIG. 6. Scheme for the isolation and determination of TMV protein in milligram lots of infected tissue.

Since the basis of expected virus yield in these experiments is the determined virus content of the homogenate used to make the experimental dilutions, the above results do not indicate the completeness with which the actual virus present in the tissue itself is recovered. As a means of testing this factor, various amounts of a purified TMV prep-

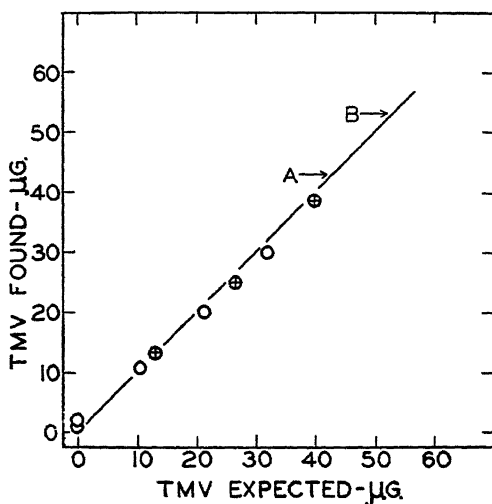


FIG. 7. TMV protein found in various artificial mixtures of homogenates from healthy and infected leaves. Two sets of data are represented. In each, homogenates representing 50 mg. of tissue/ml. were prepared from infected and healthy tissue and mixed in the proportions: 100% infected, 0% healthy; 75% infected, 25% healthy; 50% infected, 50% healthy; 25% infected, 75% healthy; 0% infected, 100% healthy. Each mixture was 1 ml. in volume, and was analyzed according to the method shown in Fig. 6. The TMV content found in the undiluted infected homogenates are represented by points A and B. The TMV expected was calculated from these values and the dilutions used. Open circles represent dilutions of homogenate A; the other points represent dilutions of homogenate B.

aration were added to an artificial mixture of equal volumes of equivalent homogenate of infected and healthy leaf tissue. Each sample was then analyzed in the usual way. As can be seen from Fig. 8, added purified TMV is recovered, but with an accuracy less than that shown in Fig. 7. The standard deviation of virus found from virus expected is $\pm 12\%$. The fact that recovery of added purified virus is significantly

less reproducible than recovery of the naturally-occurring TMV, may be due to some alteration in virus properties induced by the method of purification.

This difference in the behavior of unpurified virus material and TMV isolated by precipitation at its isoelectric point is also reflected in the data of Fig. 4. Apparently TMV becomes irreversibly aggregated when

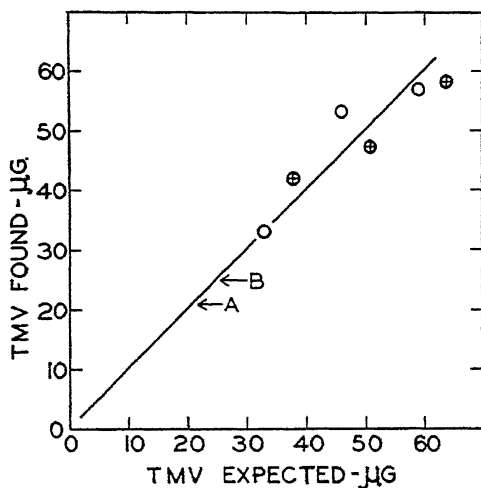


FIG. 8. The recovery of various amounts of purified TMV protein added to artificial mixtures of 50% infected, 50% healthy homogenate. Two sets of data are represented: Points A and B are the determined TMV contents of the two mixtures used. The open circles represent TMV found in homogenate A to which 13, 26, and 39 μ g. of TMV had been added. The other points represent TMV found in homogenate B to which the above amounts of purified TMV had been added. All preparations were analyzed according to the method shown in Fig. 6.

once brought to pH 3.4, and is therefore more readily precipitated by centrifugation at pH 7.0 or 4.7. However, since the virus present in infected tissue is not aggregated to this extent, this factor does not influence the yield of TMV in the analytical procedure, which according to the data cited above is of the order of 90%.

As a check on the completeness of the initial extraction of virus from the homogenized tissues, the residues from this step were re-extracted

and analyzed for TMV. The yield was 5% of the virus obtained from the first extraction.³

It is apparent from the recovery experiments cited above that the microanalytical method is a reliable means of obtaining accurate estimations of the virus content of small masses of tissue. The determination of the virus isolated by the procedure appears to be quantitative, the error involved being of the order of $\pm 5\%$. The isolation appears to yield about 90% of the total recoverable virus protein. The minimum over-all reliability of the determinations is of the order of $\pm 12\%$.

III. The Rate of TMV Synthesis in Leaf Tissue

Healthy tobacco leaves of comparable age were removed from plants, washed with sterile distilled water (and in some cases with 70% ethanol), and inoculated by rubbing with a gauze pad soaked in a solution containing 200 $\mu\text{g.}/\text{ml.}$ of purified TMV in pH 7.0 phosphate buffer. As controls, comparable leaves were rubbed with pure phosphate buffer. The leaves were kept under bell jars with their petioles in water for 18 hr. at 24°C. and 100–120 ft.-candles of illumination (from fluorescent lamps). The leaves were then washed again with sterile distilled water, and punches (each 113 sq. mm. in area) taken from the blade tissue under sterile conditions. Six to eight of these punches were placed in each of a series of sterile Petri dishes containing 15 ml. of half-strength Vickery's solution (13) and maintained at 24°C. and 100–120 ft.-candles. Periodically, single dishes were removed and smaller punches (50 sq. mm.) taken from the center of each of six punches. These were weighed

³ The efficiency of virus extraction obtained with this procedure compares closely with that obtained by Bawden and Pirie (3) by repeated digestion of minced leaf fiber with enzymes from the digestive tract of a snail. For systemically-infected tobacco leaves, they report a total TMV content of the order of 100 mg./g. of washed, dry, leaf fiber. Assuming a dry weight of 10% and taking into consideration the fact that the washing of minced leaf must remove at least 30% of its dry weight [see Frankenburg (6)], this value becomes 6.8 mg. of TMV/g. wet weight of leaf tissue. Our extraction procedure gives an average TMV content (of systemically infected leaf tissue) of 3 mg./g. wet weight, when the extraction buffer is 0.5 *M* with respect to sucrose. Since this medium does not permit chloroplast rupture, this value represents the extra-chloroplast TMV-content of the leaf. If sucrose is omitted from the extraction buffer, the chloroplasts rupture during homogenization, and the virus obtained is 50% greater than that found when sucrose is used. Hence our extraction procedure indicates an average TMV content of systemically infected leaves of 4.5 mg./g. wet weight; this approximates Bawden and Pirie's value of 6.8 mg./g. wet weight.

and analyzed for TMV content by the method outlined in Fig. 6. The results obtained from two inoculated leaves, and three phosphate-rubbed leaves are shown in Fig. 9. The virus content is expressed as $\mu\text{g.}/\text{unit leaf area}$ (302 sq. mm.) since the wet weight varies during the course of the experiment due to changes in water uptake by the tissue.

It is apparent that the method of analysis suffices to describe the rate of virus synthesis in inoculated leaf tissue. The protein obtained from uninfected leaves remains at 1–2 $\mu\text{g.}/\text{unit area}$ (zero, within the experimental error) during the entire course of the experiment. On the other hand, all virus-inoculated leaves yield rapidly increasing amounts of virus after about 72 hr.

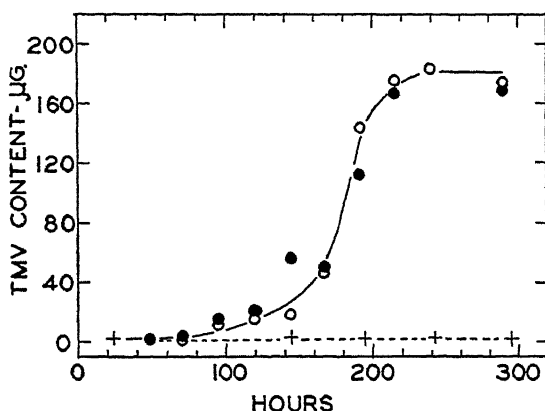


FIG. 9. The time-course of TMV synthesis in punches taken from tobacco leaf tissue. Open circles represent punches taken from a single leaf inoculated by rubbing with purified TMV at time zero; closed circles represent data for a second leaf similarly treated. Plus signs are average values of punches taken from three separate leaves rubbed with phosphate buffer at time zero. All points represent analyses of 302 sq. mm.-leaf area according to the method described in Fig. 6. The punches were maintained in one-half strength Vickery's solution at 24°C. under constant illumination.

While the curve tends to resemble the classical exponential curve of reduplication, we do not feel that the present data comprise sufficient evidence for the conclusion that TMV is produced by a so-called process of "self-duplication." Considerably more detailed analysis of the rates are necessary before such a characterization of virus synthesis could be proposed or rejected.

The data of Fig. 9 also suggest that the initiation of virus production

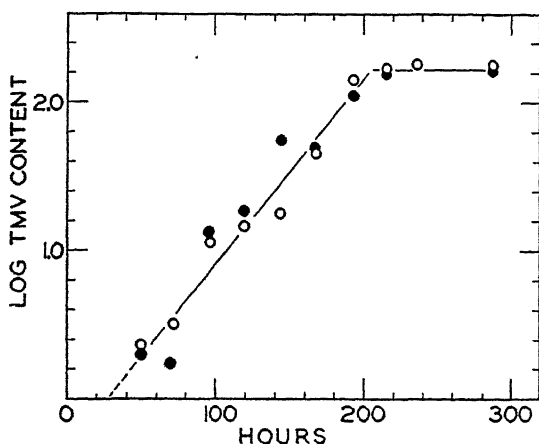


FIG. 10. Logarithmic plot of the curve shown in Fig. 9.

begins at or very soon after the time of inoculation. This can be seen more clearly from a logarithmic plot of the time-courses (Fig. 10). This plot is a straight line during the time of rapid virus formation and thereby expresses the exponential character of the course of TMV production. If the slope is extrapolated to zero TMV content, the intercept occurs at 30 hr. after inoculation. Thus, although detectable amounts of TMV are found only after about 3 days [cf. Wildman, Cheo, and

TABLE II
Biosynthesis of Virus in Leaf Punches

| Leaf | Maximum virus content: $\mu\text{g.}/302 \text{ sq. mm.}$ leaf area | Log rate: increase/24 hr. | Apparent time of initiation: after inoculation |
|------|---|------------------------------|--|
| A | 160 | 1.8 | hr. 28 |
| B | " | 1.8 | 60 |
| C | " | 2.0 | 50 |
| D | 170 | 2.0 | 30 |
| E | 180 | 2.0 | 30 |
| F | 180 | 2.0 | 24 |
| G | 161 | 2.4 | 18 |
| H | " | 2.0 | 48 |

^a Virus content still increasing at last determination, but maximum value below 160 $\mu\text{g.}$

Bonner, (16)], it is likely that TMV is present earlier, and that the initiation of the synthetic process actually occurs very soon after inoculation.

The results shown in Fig. 9 are typical of the curves of virus synthesis obtained with punches derived from a series of eight tobacco leaves. The characteristics of all eight curves (given in Table II) are quite similar. The maximum amount of virus produced (per 302 sq. mm. of leaf tissue) varies from 160 to 180 $\mu\text{g.}$; the approximate time of initiation (intercept of the log plot at zero virus content) varies from 18 to 60 hr.; the logarithmic rate factors vary from 1.8 to 2.4/day.

IV. Discussion

The above description of the course of virus production in tobacco leaf tissue provides some preliminary characterizations of the biosynthetic process. It is significant that the values typical of the curves obtained from isolated leaf tissue agree with corresponding values derived from the behavior of whole leaves or entire plants.

Thus we find that analysis of leaf blades from systemically infected plants (by the method described) gives TMV contents of 120–180 $\mu\text{g.}/302$ sq. mm. of tissue; this agrees with the maximum TMV contents cited in Table II. Similar values have been obtained from determinations of the virus content of entire leaves made by analyzing blade punches removed at various intervals after inoculation. These similarities suggest that cessation of virus production in both newly-infected tissue and in tissue which develops from infected primordia is brought about by the same or similar factors.

While the apparent lag period between inoculation and the initiation of virus synthesis is the most variable aspect of the data, its value corresponds to observations on intact plants. Thus, Cook (5) cites 18 hr. as the most rapid time in which symptoms may be detected in plants inoculated with tobacco mosaic virus, and the average time is of the order of 30 hr. These periods are within the range of the intercepts reported in Table II.

Apparently the course of virus synthesis in isolated leaf tissue corresponds to the characteristics of the process in intact leaves and whole plants. The application of the microanalytical method to such experimental material may lead to considerable information on the biochemical mechanisms related to the reduplication of tobacco mosaic virus, and may cast some light on the more general problems of the cellular role of nucleoproteins. Such investigations are now under way.

ACKNOWLEDGMENTS

We wish to acknowledge the technical assistance of Mr. Wray Darr and Mr. Leslie Paleg in certain of the experiments reported above.

SUMMARY

1. The development of a new micro method for the quantitative determination, as protein, of the tobacco mosaic virus (TMV) content of 10–50 mg. of infected tissue is described. Accurate determinations of a minimum of about 10 μ g. of virus are possible.

2. The accuracy of the method has been tested by recovering virus added to leaf homogenates. Virus added as homogenate of infected tissue can be recovered to within $\pm 5\%$ of the amount added. Purified TMV can be recovered to within $\pm 12\%$ of the amount added.

3. The method has been applied to the determination of the time-course of TMV synthesis in isolated sections of tobacco leaf blade. The appearance of TMV after inoculation of leaf tissue follows an exponential curve. Initiation of virus synthesis usually occurs within 30 hr. after inoculation. Detectable amounts of virus are recovered about 72 hr. after inoculation, and a rapid increase in virus content occurs between about 100 and 200 hr. after inoculation. During this period, the virus content doubles in approximately 24 hr. Thereafter, the virus content ceases to increase. The maximal amounts of virus produced under these conditions are similar to the TMV content of corresponding amounts of leaf tissue from systemically-infected plants.

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The Hydrolysis of Chondroitin Sulfate by Testicular Hyaluronidase¹

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INTRODUCTION

Extracts containing testicular hyaluronidase were reported several years ago to hydrolyze the chondroitin sulfate of hyaline cartilage (Ch. S.-A).³ Whether the hydrolysis was due to an enzyme distinct from hyaluronidase was left undecided, for hyaluronidases obtained from pneumococcus, streptococcus, and leech did not hydrolyze Ch. S.-A (1). On the other hand, the activity of testicular hyaluronidase towards hyaluronate appeared to be proportional to its activity towards Ch. S.-A (2). Similar results were reported by Humphrey (3).

Because of the importance of understanding the specificities of the hyaluronidase systems, the problem of the hydrolysis of Ch. S.-A was studied anew. In this study, in addition to the measurement of reducing-sugar liberated, use was made of a turbidimetric procedure for the determination of Ch. S. similar to that developed for hyaluronate. A constancy of the ratio of enzyme activity on Ch. S.-A. to that on hyaluronate in enzyme preparations obtained by different procedures and having different activities would indicate the identity of the enzyme responsible for the two activities. Preliminary turbidimetric experiments gave ratios varying from 15 to 37 (2). However, this inconstancy

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² With the technical assistance of Toby G. Bernstein.

³ In this paper we shall designate the chondroitin sulfate of hyaline cartilage (abbreviated to Ch. S.) by the letter *A* in order to distinguish it from that of skin, designated by the letter *B*. Ch. S.-*A* as a neutral salt has a specific rotation of approximately -30° and is hydrolyzed by testicular hyaluronidase preparations. Ch. S.-*B* has the general composition of *A*, but has a rotation of approximately -55° and is resistant to hydrolysis by testicular hyaluronidase.

of the ratios has now been shown to be due to experimental error, and ratios constant within much narrower limits are here reported.

EXPERIMENTAL

Materials

Three different batches of Ch. S.-A were prepared from bovine tracheal cartilage using either BaCl_2 or CaCl_2 as extractants (4). One of these samples was prepared from cartilage powder (Wilson)⁴ as the sodium salt from the barium salt (No. 21A). The other two were prepared as calcium salts from ground bovine trachea dried with acetone and ether.

Methods

Hyaluronidase activity was measured turbidimetrically by a modification of the method of Kass and Seastone⁵ (6). We have employed the modification of Tolksdorf *et al.* in which the diluted, acidified horse serum is immersed in boiling water (7). Turbidity curves obtained from a large number of batches of hyaluronate from various sources were constant to $\pm 10\%$.

TURBIDIMETRIC METHOD FOR THE MEASUREMENT OF CHONDROITIN SULFATE HYDROLYSIS

Standard Curve

A series of tubes were set up containing varying amounts (0.05–0.50 ml.) of a solution of Ch. S. in an appropriate concentration (0.600 mg./ml. of sample No. 50, and 0.900 mg./ml. of sample Nos. 21 and 71) in 0.1 M pH 6.0 acetate buffer containing 0.15 M NaCl. An amount of the same buffer was added to each tube to make a total of 1 ml. The "blank" contained 1 ml. of the buffer. To each tube were added 3 ml. of 0.5 M pH 4.2 acetate buffer and 1 ml. of diluted serum, prepared as follows: normal horse serum was diluted with 9 volumes of 0.5 M pH 4.2 acetate buffer and then acidified to pH 3.1 with 4 N HCl; immediately before use the solution was heated for 30 min. in a boiling water bath, cooled, and filtered. Each tube was shaken and allowed to stand at room temperature. After 30 min., the resulting turbidities were measured in a Coleman model No. 11 spectrophotometer at wavelength 580 m μ , employing Aminco cuvettes No. 5–950 (10 \times 25 mm.) in a specially constructed adapter. The tubes were compared with the blank which was set at 100% transmission. The standard curve was made by plotting log per cent transmission against milligrams of Ch. S.

Two samples, Nos. 21 and 71, gave similar turbidity curves while sample No. 50 precipitated larger amounts of protein. This difference

⁴ We wish to thank Dr. David Klein of the Wilson Laboratories for the generous gift of this material.

⁵ In a recent review (5), the substrate concentration was erroneously printed as 4 mg./ml. instead of 0.4 mg./ml.

TABLE I
Analysis of Chondroitin Sulfate Preparations

| Preparation | N | Hexos- amine | Uronic acid | Sulfate | $[\alpha]_D^{20}$ | Initial conc. used for turbid- imetry |
|--|-----------------|-----------------|-----------------|-----------------|-------------------|--|
| | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>degrees</i> | <i>μg./ml.</i> |
| 21B Wilson carti- lage powder, Na salt | 2.97 | 27.7 | 29.7 | 15.6 | -26 | 450 |
| 50B Bovine trachea Ca salt | 3.09 | 25.1 | 29.9 | 12.6 | -24 | 300 |
| 71 Bovine trachea Ca salt | 2.79 | 24.4 | 32.2 | 16.1 | -28 | 450 |

necessitated using different initial concentrations of Ch. S. in the experiments.

In Table I the analytical data of the three samples are listed.

Nitrogen was determined by micro-Kjeldahl.

Hexosamine was determined after 12 hr. hydrolysis with 4 *N* HCl (8), the color being measured in a Coleman model 11 spectrophotometer, using 50-mm. cuvettes and a wavelength of 530 μ .

Uronic acid was determined by the method of Tracey (9).

Sulfate was determined according to Alicino (10) employing a modification of the procedure of Meyer *et al.* (11). A 10-mg. sample was hydrolyzed by refluxing for 2 hr. with 5 ml. 1 *N* HCl. The solution was evaporated to dryness at room temperature in a vacuum desiccator over NaOH. The residue was taken up in 2.5 ml. water, neutralized to phenolphthalein as internal indicator, and diluted with 2.5 ml. 95% ethanol. The solution was then titrated with 0.02 *M* BaCl₂ in the presence of several drops of an aqueous solution of potassium rhodizonate.

Activity Measurement

One-half ml. of the standard Ch. S. solution was added to each of a series of tubes containing appropriate dilutions of enzyme in 0.5 ml. of 0.1 *M* pH 6.0 acetate buffer containing 0.15 *M* NaCl. The tubes were shaken and immediately placed in a 37° water bath where they were incubated for 30 min. They were then heated to 60° in a water bath (running, hot tap water) for 10 min. to inactivate the enzyme. Then 3 ml. of pH 4.2 buffer and 1 ml. of diluted horse serum (same as for standard curve) were added to each tube and the tubes allowed to stand for 30 min.; the turbidities were then measured as described above. The quantity of enzyme producing "half turbidity" (*i.e.*, the turbidity given by half the initial Ch. S. concentration) is read from the plot of the log per cent transmission against milligrams of enzyme, and represent the enzyme activity. One turbidity-reducing unit (abbreviated in the table to TRU) is

defined as the quantity of enzyme which causes in 30 min. a reduction in the turbidity to that given by half the initial substrate concentration.

Table II gives the activities of a series of testicular hyaluronidase preparations towards hyaluronate and Ch. S. and the ratio of these activities as obtained with the three Ch. S. samples.

It can be seen from Table II that ten different enzyme preparations obtained from bull and ram testes by a variety of methods including salt fractionation, lead precipitation, and adsorption on bentonite show constant ratios of the two activities within the limits of the experimental method.

It should be noted that the apparent enzyme activity is a function of the substrate concentration. This is to be expected from the fact that

TABLE II
*Depolymerization (Turbidity Reduction) of Chondroitin Sulfate
by Testicular Hyaluronidases^a*

| Enzyme | Method of prep. | Activity on hyaluronate | Activity on chondroitin sulfate | | | Activity ratio Chondroitin sulfate Hyaluronate | | |
|---------------------|-----------------|-------------------------|-------------------------------------|-------------------------------------|-------------------------------------|--|--------|--------|
| | | | CSA 21 | CSA 71 | CSA 50 | CSA 21 | CSA 71 | CSA 50 |
| | | TRU ^c /mg. | TRU ^c _{ch} /mg. | TRU ^c _{ch} /mg. | TRU ^c _{ch} /mg. | | | |
| W179A1 ^b | | 1200 | 66 | 65 | 172 | 0.055 | 0.054 | 0.14 |
| 43CII | <i>d, e</i> | 710 | 54 | 45 | 92 | 0.076 | 0.063 | 0.13 |
| 14B | <i>d, e</i> | 630 | 38 | 29 | 86 | 0.060 | 0.046 | 0.14 |
| 25A | <i>d</i> | 260 | 12 | 20 | 26 | 0.046 | 0.077 | 0.10 |
| 25B | <i>d</i> | 250 | 14 | 16 | 28 | 0.056 | 0.064 | 0.11 |
| 38H | <i>d, f</i> | 220 | 11 | 15 | 33 | 0.050 | 0.068 | 0.15 |
| 14A | <i>d, e</i> | 500 | | | 82 | | | 0.16 |
| 43CI | <i>d, e</i> | 480 | | | 78 | | | 0.16 |
| 38E | <i>d, e</i> | 230 | | | 32 | | | 0.14 |
| 18B ^a | <i>e, g</i> | 105 | | | 14 | | | 0.13 |
| | | | | | Mean | 0.057 | 0.062 | 0.14 |

^a 18B was prepared from ram testis. The source of the other preparations was bull testis.

^b We are greatly indebted to Dr. Joseph Seifter of the Wyeth Institute, Philadelphia, for this sample.

^c Turbidity-reducing unit.

^d Ammonium sulfate fractionation.

^e Lead acetate fractionation.

^f Adsorption on bentonite and elution with pyridine buffer.

^g Acetone precipitation.

TABLE III
*Hydrolysis (Reducing Sugar Formation) of Chondroitin Sulfate
 by Testicular Hyaluronidases*

| Enzyme | Hyaluronidase activity | Activity ratio ^a $\frac{\text{Chondroitin sulfate}}{\text{Hyaluronate}}$ | | |
|--------|------------------------|---|--------|--------|
| | | CSA 21 | CSA 71 | CSA 50 |
| W179A1 | TRU ^b /mg. | | | |
| 43CII | 1200 | 1.4 | 1.6 | 1.5 |
| | 710 | 1.5 | 1.6 | 1.5 |

^a Extent of formation of glucose equivalent in 120 min. For details see text.

^b Turbidity-reducing unit.

with higher initial substrate concentrations (CSA 21, CSA 71), more substrate must be hydrolyzed to obtain "half turbidity."

Reductimetric Comparison

The experiments were set up so that the rate of reducing sugar formation was approximately the same with both hyaluronate and Ch. S. To achieve this effect, Ch. S. at a concentration of 3.0 mg./ml. was incubated at 37° with enzyme at a concentration of 0.5 mg./ml., while hyaluronate was employed at 1.0 mg./ml. with the enzyme at 0.05 mg./ml. The substances were dissolved in 0.1 M acetate buffer pH 5.0 containing 0.15 M NaCl. Aliquots of 0.25 ml. were withdrawn from the incubation mixture at intervals of 60 and 120 min. Reducing sugar was determined by the Hagedorn-Jensen method. Enzyme and substrate controls remained unchanged. An increase of 50–100 μ g. of glucose equivalent was measured in the aliquot for the 120-min. period. Comparison of the extent of hydrolysis of the three Ch. S. preparations with that of hyaluronate by the two most active enzymes showed the relative activities to be identical.

The pH optimum for Ch. S. hydrolysis as determined reductimetrically was found to be 4.7. At pH 6.0, the pH of the turbidimetric experiments, the rate of formation of reducing sugar was approximately 60% of the maximum.

ENZYME ACTION ON CARTILAGE POWDER

It seemed interesting to determine whether testicular hyaluronidase was able to hydrolyze Ch. S. as it occurs in the tissue.

Finely ground and dehydrated cartilage powder (Wilson) containing 11.0% hexosamine was incubated at 37.0° as a suspension at a concentration of 12.0 mg./ml. with 1.0 mg./ml. of a testicular hyaluronidase (W179AI) in the buffer mixture described

above, at pH 5.0, with toluene as a preservative. The increase in reducing sugar (expressed as glucose equivalent) in the supernatant solutions corresponded in 2 hr. to 190 $\mu\text{g.}/\text{ml.}$ and in 24 hr. to 340 $\mu\text{g.}/\text{ml.}$ A control experiment without enzyme showed an increase of 100 $\mu\text{g.}/\text{ml.}$ of glucose equivalent in 24 hr. These results are in agreement with those of Humphrey (3).

DISCUSSION

The constancy of the ratio of enzyme activity on Ch. S.-A to activity on hyaluronate, determined under comparable conditions, with a variety of testicular enzyme preparations from bull and ram testes, strongly suggests the identity of the two enzymatic functions. The absence of catalysis of Ch. S. hydrolysis by pneumococcal, streptococcal, and leech hyaluronidases emphasizes the differences between the various enzyme systems.

Knowledge of the enzymatic breakdown of Ch. S. in the animal body is very meager. As was pointed out earlier (5) the reaction product of hyaluronidase on Ch. S. appears to be a sulfated disaccharide. Whether the sulfate groups are removed enzymatically in the mammalian tissues, as is reported for certain bacteria (12), still remains to be determined. Although the turnover of Ch. S. in hyaline cartilage is presumably quite small, it may be considerable in callus and in the calcifying epiphysis. It remains to be established whether chondroitin sulfate is removed from these tissues and by what mechanism this may be accomplished. The presence of a chondroitin sulfate in urine has been reported by Mörner (13), but lack of characterization precludes interpretation with regard to metabolism of this substance.

The enzymatic hydrolysis of Ch. S.-A in cartilage powder by testicular hyaluronidase contrasts sharply with the repeatedly reported failure of the enzyme to affect the staining reactions of sections of hyaline cartilage (14,15). Whether this failure is due to nonpenetration of the enzyme or to other circumstances is not known. In any case, the experiments emphasize the difficulties of interpretation of histochemical observations.

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SUMMARY

A turbidimetric method for the assay of chondroitin sulfate-A is described. Three samples of chondroitin sulfate-A were prepared as either the sodium or calcium salts. The turbidimetrically determined depolymerization of these substrates by a number of testicular hyaluronidase preparations obtained from bull and ram testes by a variety of procedures and varying in hyaluronidase activity between 1200 and 100 turbidimetric units/mg. was studied. The ratio of activity toward both substrates was constant under comparable conditions. The same constancy of the ratio was found when the hydrolysis was measured reductimetrically. The constancy of these ratios indicates that the hydrolysis of both chondroitin sulfate-A and hyaluronate is attributable to the same enzyme.

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Inhibitory Action of Diamidines and Stimulative Effect of Polyamines on Enzymatic Activities of *Escherichia coli* and *Micrococcus aureus*

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INTRODUCTION

The antibacterial activity of diamidines first noted by Fuller (1) has been confirmed by a number of investigators (2,3,4). Bichowsky-Slomnitzki (5) has described the inhibitory effect of stilbamidine and pentamidine on the growth of *Escherichia coli* and *Micrococcus aureus* and its antagonism by a number of agents. Experiments reported in the first part of this paper deal with the effect of diamidines on enzyme systems of these organisms and its counteraction by polyamines. In the course of these investigations the observation was made that polyamines may stimulate dehydrogenase activities. Experiments reported below define conditions for the demonstration of this effect and contribute to an understanding of its nature.

METHODS

After several passages through broth containing 0.2% of substrate, the bacteria were grown for 20 hr. at 37°C. on agar of a similar composition. They were then taken up in saline and washed several times. The cell suspensions contained about 1.8×10^{10} cells/ml. *M. aureus* suspensions rapidly lost activity on storage for more than 1 day in the refrigerator. Fresh cell suspensions of this organism were used. Cell suspensions of *E. coli* could be kept in the cold without marked loss of activity for 5 days. Cells grown on 0.2% glucose agar were used in the preparation of extracts. The washed cells were frozen with Dry Ice snow and thawed at 37°C. After 5 or 6 freezings they were held for 3 days at 37°C. in thick suspensions with an addition of thymol. The opalescent fluids obtained by centrifugation were examined for dehydrogenase activity.

Aromatic diamidines were kindly provided by Messrs. May and Baker Ltd. Solutions of the stilbamidine and pentamidine chlorides were prepared in distilled water immediately before use. Three synthetic polyamine chlorides were kindly provided by

Carbon and Carbide Company, New York: diethylenetriamine (DT), triethylenetetramine (TT), and tetraethylenepentamine (TP). Spermine phosphate (SP) was kindly provided by Hoffmann-La Roche, Basel, Switzerland. Additional experiments were carried out with ethylenediamine (ED), phenylenediamine (PD), and hexamethylenediamine (HMD).

Dehydrogenase activity was determined with Thunberg's method using the modification described by Braun and Woerdenhoff (6) at 38°C. The reaction mixtures contained 0.1 ml. of methylene blue solution 1:10,000, 1.0 ml. of 0.2% substrate solution, 1.0 ml. of bacterial suspension or extract, and 1.0 ml. of saline solution under a seal of 5.0 ml. vaseline in a test tube. Respiration and glycolysis rates were determined manometrically with the Warburg apparatus at 37°C. Respiration was determined in mixtures made up of 1.0 ml. cell suspension, 1.0 ml. 0.1 *M* substrate solution, and 1.0 ml. phosphate buffer of pH 7.4. Glycolysis was measured in mixtures containing 1.0 ml. cell suspension, 1.0 ml. 0.1 *M* glucose solution, and 1.0 ml. of bicarbonate-salt solution of Krebs (7) under a gas mixture of 95% nitrogen and 5% carbon dioxide.

Desamination was measured with Conway's microdiffusion technique (8) at 37°C. One ml. of cell suspension with 0.5 ml. 0.05 *M* solution of amino acid were placed in the main chamber and 1.0 ml. of dilute boric acid solution in the central chamber. The reaction was stopped after 2 hr. with 1.0 ml. of concentrated potassium carbonate solution. The amount of liberated ammonia was measured 2 hr. later.

Pyruvate and succinate form a precipitate with diamidines. The action of diamidines on the metabolism of these substrates was therefore tested in saline medium on cells which had been pretreated with diamidine for a definite interval.

RESULTS

Inhibition of Dehydrogenases by Diamidines and Its Counteraction by Polyamines

Diamidines caused a considerable reduction in dehydrogenase activity. As may be seen in the experiments reported in Table I, the inhibition was largest with pyruvic dehydrogenase and least with formic dehydrogenase.

Diamidines failed to inhibit dehydrogenase activity in the presence of adequate amounts of polyamines (see Table I). Triethylenetetramine protected the dehydrogenase systems of *E. coli* and *M. aureus* when its molar concentration was 10 times that of the diamidines. Reactivation of the inhibited enzymes was observed with TT when it was added to cells previously exposed to diamidines. The action of a 0.001 *M* solution of the diamidines was antagonized by a 0.01 *M* solution of DT, TT, and TP. Diethylenetriamine was the least effective of the three. Experiments with TT confirmed that this substance fails to exert a toxic effect on the microorganisms even at the highest concentrations in which it was used. Spermine phosphate, a naturally occurring polyamine, failed to antagonize the action of the diamidines.

TABLE I
*Inhibition of Bacterial Dehydrogenases by Diamidines and
 Antagonism by Polyamines*

| Microörganism examined | Substrate | Decoloration time of methylene blue in minutes by | | | | | |
|---------------------------|-----------|---|------------------------|-----------------------------|------------------------|-------------------|-----------------------|
| | | Cells exposed for 1 hr. to the action of | | | | | Untreated controls |
| | | Pentamidine $10^{-3} M$ | | Stilbamidine $10^{-3} M$ | | TT $10^{-2} M$ | |
| | | Without TT | With TT $10^{-2} M$ | Without TT | With TT $10^{-2} M$ | | |
| <i>E. coli</i> | Lactate | 25 | | 14 | | | 10 |
| | Formate | 15 | | 12 | | | 12 |
| | Glucose | 55 | 25 | 69 | 17 | 17 | 18 |
| | Pyruvate | 125 | 40 | 143 | 30 | 14 | 14 |
| | Succinate | 45 | 20 | 40 | 16 | 10 | 10 |
| <i>M. aureus</i> | Lactate | 15 | | 15 | | | 11 |
| | Formate | 25 | | 10 | | | 10 |
| | Glucose | 85 | 20 | 27 | 20 | 18 | 20 |
| | Pyruvate | 200 | 45 | 55 | 35 | 30 | 30 |

Under the conditions used, extracts of *E. coli* caused decoloration of methylene blue by glucose in 30 min. In the presence of $10^{-3} M$ stilbamidine and pentamidine, the decoloration times with glucose were 56 and 42 min., respectively.

*Inhibitory Action of Diamidines on Respiration and
 Its Counteraction by Polyamines*

Suspensions exposed to the action of stilbamidine or pentamidine showed a marked diminution in oxygen consumption, pentamidine being the most effective of the two in this respect.

The action of the amidines on respiration was antagonized to varying degrees by the polyamines. A complete counteraction of inhibition by diamidines was not obtained with TT even when its concentration was raised to 0.1 *M*. Results with *M. aureus* and *E. coli* were rather similar. The effect of stilbamidine was somewhat less with the former of the two organisms.

*Inhibition of Glycolysis by Diamidines and
Antagonism by Polyamines*

Suspensions exposed to 0.001 *M* diamidine solutions showed a fall in glycolytic activity ranging between 50 and 80%. The effects on glycolysis paralleled those on respiration. Polyamines abolished the inhibitions partially or completely. The results are summarized in Table II.

*Inhibition of Desamination by Diamidines and
Antagonism by Polyamines*

The effect of diamidines on desamination of selected amino acids is illustrated by the experiments summarized in Table III. Diamidines in

TABLE II
*Antagonism of Polyamines to Inhibiting Action of Diamidines on
Glycolysis by E. coli and M. aureus*

| Microorganism examined | Glycolysis during 2 hr., as CO ₂ by | | | | |
|---------------------------|--|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------|
| | Cells exposed to | | | | Untreated cells |
| | Stilbamidine 10 ⁻³ <i>M</i> | | Pentamidine 10 ⁻³ <i>M</i> | | |
| | Without TT | With TT, 10 ⁻² <i>M</i> | Without TT | With TT, 10 ⁻² <i>M</i> | |
| <i>E. coli</i> | mm. ³ 70.5 | mm. ³ 160.0 | mm. ³ 41.5 | mm. ³ 208.0 | mm. ³ 213.0 |
| <i>M. aureus</i> | 35.0 | 99.0 | 40.0 | 177.0 | 136.5 |

a concentration of 5×10^{-4} *M* caused a marked decrease in the activity to L-alanine and leucine, lesser effects in the activity to aspartic acid, and only a slight effect on the activity to arginine. Polyamines reduced the inhibitory effects yet failed to restore normal values. A ten-fold increase of the polyamine concentration did not change this result.

*Influence of Aging and Washing on Dehydrogenase Activity and the
Restoration of the Activity by Polyamine Addition*

The reduction of dehydrogenase activity by aging and the effect of polyamines on the dehydrogenase activity of the fresh and aged cell

TABLE III
*Antagonism of Polyamines to Inhibiting Action of Diamidines on
 Desaminating Activities of E. coli*

| Amino acids examined | Ammonia evolved within 2 hr. by | | | | Untreated cells |
|----------------------|---------------------------------|----------------------|-------------------------|----------------------|-----------------|
| | Cells exposed to | | | | |
| | Stilbamidine 10^{-3} M | | Pentamidine 10^{-3} M | | |
| | Without TP | With TP, 10^{-2} M | Without TP | With TP, 10^{-2} M | |
| L-Alanine | mg. 27 | mg. 44 | mg. 10 | mg. 29 | mg. 57 |
| Glycine | 24 | 39 | 12 | 25 | 49 |
| Aspartic acid | 90 | 112 | 44 | 49 | 105 |
| Glutamic acid | 24 | 28 | 20 | 34 | 44 |

suspensions are illustrated by the experiments summarized in Table IV. It is apparent that polyamines exert only a slight effect on the dehydrogenase activity of the freshly harvested cells. Cells with dehydrogenase activity diminished by aging for 14 days in the refrigerator were stimulated by the polyamines to increased activity with respect to pyruvate, glucose, and succinate. On the other hand, lactic and formic dehy-

TABLE IV
Action of Polyamines on the Dehydrogenases of E. coli

| Substrate | Decolorization time in min. by | | | | | | | | |
|-----------|--|---------|--|-------------|-------------|-------------|-------------|-------------|---------|
| | Fresh suspensions at a concentration of TP | | Fourteen-day old suspensions at concentrations of TP | | | | | | |
| | 10^{-3} M | Control | 10^{-3} M | 10^{-2} M | 10^{-4} M | 10^{-5} M | 10^{-6} M | 10^{-7} M | Control |
| Glucose | 14 | 14 | 20 | 20 | 20 | 20 | 32 | 48 | 50 |
| Pyruvate | 22 | 25 | 32 | 32 | 33 | 32 | 35 | 65 | 82 |
| Succinate | 25 | 25 | 30 | 30 | 30 | 30 | 30 | 33 | 50 |
| Lactate | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 20 | 20 |
| Formate | 28 | 28 | 30 | 30 | 30 | 30 | 30 | 30 | 30 |

Experimental conditions: 1 ml. of bacterial suspension, 0.5 ml. methylene blue diluted 1:10,000, 0.5 ml. TP, 0.5 ml. of 0.1 substrate, 1 ml. phosphate buffer pH 7.4, saline to ml. The organisms were exposed to polyamine 15 min. before addition of substrate.

drogenases of *E. coli* were little affected by aging and showed no response to the polyamines.

An experiment on cell suspensions of *M. aureus* is presented in Table V. It may be seen that TP, TT, and DT stimulate the dehydrogenase activity of the washed *M. aureus* cell, and that the other diamines fail to exert this effect. Phenylenediamine (PD) exerted a slight retardant action. Under these conditions, thiamine induced but a small increase in dehydrogenase activity. Riboflavin, calcium pantothenate, pyridoxin, and biotin proved to be without effect.

TABLE V

The Action of Polyamines and Diamidines on the Dehydrogenases of M. aureus

| Substrate | Decolorization time in min. | | | | | | | | | | | | |
|-----------|--|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|--------------|
| | in the presence of molar concentrations of | | | | | | | | | | | | |
| | TP | | TT | | DT | | ED | | SP | | PD | | Control tube |
| | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁵ | |
| Glucose | 40 | 27 | 40 | 27 | 40 | 28 | 65 | 60 | 70 | 70 | 81 | 72 | 70 |
| Pyruvate | 28 | 23 | 28 | 24 | 30 | 28 | 70 | 65 | 85 | 85 | 95 | 85 | 85 |
| Lactate | 23 | 20 | 23 | 20 | 24 | 20 | 30 | 30 | 32 | 32 | 41 | 30 | 33 |
| Formate | 18 | 15 | 17 | 15 | 17 | 16 | 40 | 38 | 40 | 40 | 46 | 40 | 40 |

Experimental conditions: Bacterial suspensions washed twice in saline and kept for 24 hr. in the refrigerator. Other conditions as in Table I.

Repeated washing of *E. coli* and *M. aureus* cells produced a loss in dehydrogenase activity which could be restored partially by addition of polyamine.

When cells aged by storage in the refrigerator were separated from their suspension medium by centrifugation, a further loss of dehydrogenase activity was observed (see Table VI). The supernatant fluid was concentrated threefold by evaporation. Addition of the concentrate to washed cells which had been stored for 5 days in the refrigerator resulted in marked increase of their dehydrogenase activity.

The effect of the concentrate exceeded that of the polyamines. Simultaneous addition of both polyamine and the concentrate proved less effective than the addition of the concentrate alone. The decoloration times were as follows: Control without addition of supernatant or TT,

TABLE VI

The Effect of Removing the Supernatant Fluid From Old Suspensions of E. coli on the Activity of Dehydrogenases

| Substrate | Decolorization time in min. by 12-day old suspensions | | | |
|-----------|---|----------------------|--|----------------------|
| | Left in contact with suspending fluid | | Suspending fluid removed by centrifugation | |
| | Control | TT 10^{-4} M added | Control | TT 10^{-4} M added |
| Glucose | 68 | 37 | 98 | 37 |
| Succinate | 70 | 39 | 95 | 37 |

45 min.; with addition of supernatant, 17 min.; with addition of supernatant and TT at a final concentration of 10^{-5} M, 30 min.; with TT alone, 30 min. This suggests that the polyamines are effective as activators of dehydrogenation because they have some similarity to a substance which occurs in the cell and which may be recovered from it by aging and washing.

Effect of Polyamines on Oxygen Uptake and Glycolysis

In general, polyamines exerted little effect on the oxygen consumption of fresh and aged cell suspensions in the presence of glucose and succinate, but in a few instances a parallel increase of both oxygen uptake and dehydrogenase activity towards these substrates was ob-

TABLE VII

The Stimulation in Oxidation of Pyruvate by E. coli in Presence of Polyamines

| Polyamine employed | Molar concentration | Oxygen uptake per 2 hr. | Stimulation of oxidation |
|--------------------|---------------------|-------------------------|--------------------------|
| | $\times 10^{-4}$ | mm. ³ | % |
| DT | 10 | 109 | 54 |
| TT | 100 | 132 | 86 |
| | 10 | 135 | 90 |
| | 1 | 110 | 54 |
| TP | 10 | 132 | 86 |
| SP | 10 | 70 | — |
| Control | — | 71 | — |

served in the presence of polyamine. Pyruvic dehydrogenase of *E. coli* was rapidly inactivated by aging. Polyamines restored the pyruvate oxidation activity of these cells as shown in Table VII. Tetraethylenepentamine and TT were about equally effective in this respect, whereas DT was less active. Polyamines produced a clear-cut stimulation of glycolysis and glucose dehydrogenation by the aged cells but exerted little action on their uptake of oxygen in the presence of this substrate.

DISCUSSION

It is apparent that diamidines exert marked inhibitory effects on oxidation-reductions catalyzed by *E. coli* and *M. aureus*. The action is not to be ascribed to an injury of cell structure since it is demonstrable also on cell extracts.

Bernheim (4) has reported that diamidines with antibacterial action interfere selectively with oxidation of amino acids and do not affect the oxidation of pyruvate, glucose, and succinate. The experiments summarized in the present paper suggest that diamidines are equally effective inhibitors of pyruvate and amino acid oxidation, and that high concentrations of diamidine also effect inhibition of glucose and succinate oxidation. The discrepancy between the present findings and those of Bernheim (4) is probably an outcome of differences in experimental technique. Bernheim (4) examined the action of diamidines in the presence of the substrate, whereas in our work pretreatment of the cells with diamidine was preferred in order to avoid error caused by formation of precipitates due to the interaction between the substrate and diamidine.

The antagonistic effect of polyamines on the action of atebirin and quinine on *E. coli* was first shown by Silverman and Evans (9,10). Snell (11) showed that the action of propamidine on *Lactobacilli* can be antagonized by natural and synthetic polyamines. Silverman and Evans (10) have proposed the view that polyamines play an essential role as substrates in cell metabolism and that diamidines exert an inhibitory effect by competition with cell polyamines. Elson (12) considers that the antagonism between diamidines and polyamines is nonspecific in nature [cf. (Ref. 13)]. This investigator favors the view that polyamines compete with one another for an essential cell constituent, probably a nucleic acid in view of the demonstration by Bichowsky (3) that nucleic acids are antagonists of the diamidines.

Nonspecific stimulation of oxidative systems by subinhibitive concentrations of toxic substances is a well-known phenomenon. However, the stimulating effect of the polyamines on dehydrogenation is observed uniformly over a wide concentration range. The concentrations of polyamine which cause stimulation approach those of accessory growth factors and it seems reasonable to suspect that the polyamines, like the latter, function as a coenzyme. This view is further supported by our observation that supernatant fluid of aged cell suspensions contains a dehydrogenation activator with which the polyamines compete and which they are able partially to replace. It may be noted that the coenzymes I and II concerned in dehydrogenation of glucose and lactate do not play a part in succinate dehydrogenation (14). The polyamines, however, activate the dehydrogenation of succinate.

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SUMMARY

1. *E. coli* and *M. aureus* cells exposed to the action of diamidines show marked reductions in dehydrogenase activity. Their dehydrogenase activity is also reduced by washing and aging.

2. The inhibition of dehydrogenase activity by diamidines is antagonized by synthetic polyamines. The activity lost on aging may be restored by addition of polyamines.

3. Diamidines caused inhibition of oxygen uptake, glycolysis, and desamination. Polyamines abolished these inhibitory effects incompletely.

4. Supernatant fluid of aged cell suspensions of *E. coli* contains a factor which can activate dehydrogenase activity. Polyamines compete with and partially replace this factor.

5. Polyamines accelerate aerobic pyruvate oxidation by the aged cells but fail to affect the oxidation rate of a number of other substrates. They similarly accelerate glycolysis of cell suspensions whose activity has been diminished by aging.

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An Improved Method for Determination of *p*-Aminobenzoic Acid by *Neurospora crassa*¹

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INTRODUCTION

Several methods have been proposed for the determination of *p*-aminobenzoic acid (PAB), but most of these have serious shortcomings. The chemical method of Bratton and Marshall (1) does not possess sufficient sensitivity for use with some natural materials. The method is also not specific, since a color is developed with any diazotizable arylamine. A microbiological method using *Acetobacter suboxydans* has been described by Landy and Dicken (2). This organism is an aerobe and forms a film on the surface, which is difficult to homogenize and makes the turbidity reading irregular and unreliable. Lewis (3) developed a method using *Lactobacillus arabinosus*, but the response seems to vary with the strain (4). Also, Leonian and Lilly (5) were unable to obtain satisfactory results with it because a trace of PAB left in the casein hydrolysate induced too much growth in the controls. With a stronger norite treatment to remove the last traces of PAB the organism failed to grow even in the presence of added PAB. Pennington (4) published a short note recommending the use of *Leuconostoc mesenteroides* for assay of PAB. Maximum growth was given by 0.1 m μ g./ml. of the medium and hence the assay is rather sensitive for routine use. No data on the specificity and reproducibility of the method were given. *Clostridium acetobutylicum* was used by Lampen and Peterson (6), but the assay is too sensitive for routine use and the incubation must be done under strongly anaerobic conditions. Also the PAB requirement of this organism does not exist in the presence of purine

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² Government of India Scholar.

bases (7). Thompson *et al.* (8) described an assay with a mutant of *Neurospora crassa*, in which the diameter of the mold growth on an agar base is taken as a measure of the amount of PAB in the medium. Leonian and Lilly (5) using the same mutant took the weight of mycelium as an index of the PAB content. However, growth on their medium was far below that obtained on a good natural medium. This defect might introduce a serious error in the determination when, because of low PAB content, a large weight of sample had to be used.

In the present paper the medium has been modified so as to give optimal growth of *Neurospora crassa* 1633 and the response of the test organism to pteroylglutamic acid and other related compounds has been studied in order to determine the specificity of the assay.

EXPERIMENTAL TECHNIQUE

Culture

The organism used in this study is a mutant strain of *Neurospora crassa*, which requires this vitamin and was designated by Tatum and Beadle (9) as *Neurospora crassa*, *p*-aminobenzoicless No. 1633. It was carried on slants containing 0.25% glucose, 0.25% malt extract, 0.25% yeast extract, and 1.5% agar and was transferred at monthly intervals.

Basal Medium

This mold will grow suboptimally on a medium consisting of salts, sucrose, biotin, and PAB. But for assay purpose a richer medium is desirable. The medium used here contained the following (in g./l.): ammonium tartrate 5, ammonium nitrate 1, KH_2PO_4 1, $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, NaCl 0.1, CaCl_2 0.1, glucose 25, asparagine 2.5, casein hydrolysate (PAB-free) 2. Trace elements added in mg./l. were B 0.01, Mo 0.02, Fe 0.20, Cu 0.10, Mn 0.02, Zn 2.0. The medium also contained biotin, 40 $\mu\text{g./l.}$, and PAB as required for the assay. The pH of the medium was adjusted to 5.0. This medium is the same as that used by Horowitz and Beadle (10) for choline assay by a "cholineless" mutant of this organism, except that glucose has been substituted for sucrose, and casein hydrolysate and asparagine have been added to improve growth and to remove interference from the possible stimulatory effect of amino acids that might be present in the samples. A much greater response and a smoother curve are obtained by the inclusion of these substances. The biotin content has also been increased to 40 $\mu\text{g./l.}$, so as to avoid stimulation from biotin present in samples. On this improved medium the growth was practically maximal: little or no increases were obtained by the addition of small quantities (10 mg.) of liver L, peptone, yeast extract, or a mixture of the three. It was not until large quantities (50 mg.) of these natural substances were added that the growth was increased, about 15-20% above that obtained on the original medium.

PAB-Free Casein Hydrolysate

Fifty g. of Labco "vitamin-free" casein was refluxed for 8 hr. with 500 ml. of 20% HCl. The hydrolysate was evaporated under reduced pressure to a thin sirup, 200 ml. of water was added, and the water was again removed. The addition of water and evaporation was repeated twice and the resulting sirup was dissolved in water, adjusted to pH 4.0 with NaOH, and diluted to 500 ml. It was then treated with 10 g. of Darco G-60 for 30 min. on a steam bath, cooled, and filtered. The solution was brought to pH 2.0 with H₂SO₄, continuously extracted with ether for 24 hr., and then stored in the cold room at 4°C.

PROCEDURE

The assays were run in 50-ml. Erlenmeyer flasks. Five ml. of double strength medium was pipetted into flasks (cleaned with chromic acid solution, otherwise a higher blank is obtained). The aliquots of sample after proper dilution were added and the volume was made up to 10 ml. The flasks were arranged in trays of 50 each and then sterilized for 15 min. at 15 lb. pressure. The known and unknown samples must have the same pH, as the availability of PAB to this organism depends upon pH (11). In our assays, all the samples were adjusted to pH 5.0 before use.

For inoculum purposes, a spore suspension in sterile distilled water reading about 60-65 in the Evelyn colorimeter (660 μ filter with water as 100) was made and 1 drop/flask was used as inoculum. The flasks were incubated at 30°C. for 72 hr. After incubation, the flasks were rotated with a twist, so as to break the mycelium from the sides of the flask in one solid pad. The trays were then autoclaved for 10 min. at 15 lb. to kill the mold. After this, the solid intact pads were removed with a wire needle, placed in weighed pyrex tubes, and washed twice with distilled water by means of low-speed centrifugation. The tubes and mycelial pads were dried at 109°C. for 12-16 hr. and weighed. By this procedure it was convenient to run about 150-200 flasks at one time.

Standard Curve

Under the conditions given, the assay range is 0-100 μ g. PAB/10 ml., and the weight of dry mycelium is 0-54 mg. The curve starts flattening at 100 μ g./10 ml. A standard curve is shown in Fig. 1. Also included for comparison is a curve obtained with the same levels of PAB, but without supplementing the medium with asparagine and casein hydrolysate. For each level of PAB the growth with supplements

is from two to three times greater than that without supplements. Moreover, the plateau is reached at 60 $\mu\text{g.}/\text{flask}$ without supplements as compared with 100 $\mu\text{g.}/\text{flask}$ with supplements. Thus supplementing the medium actually increases the range of the assay.

As an additional check on the assay, a reference sample of pulverized yeast was always included in each assay. Table I shows the reproducibility of the assay at different levels and also the variability at the same level. Variation at the 20 $\mu\text{g.}$ level was smaller than at higher levels. Between 40–100 $\mu\text{g.}$ the variation from low to high averaged 4.1 mg. , which is well within the range of error in microbiological assays. The average values for the reference yeast at three different

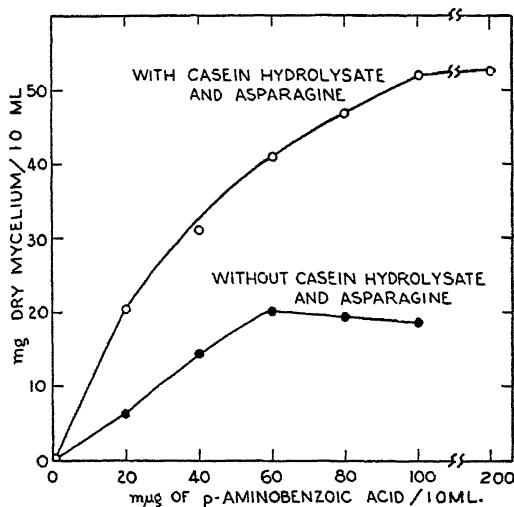


FIG. 1. Response of *Neurospora crassa* "p-aminobenzoicless" to added p-aminobenzoic acid.

levels, 1, 3, and 5 mg. , are 9, 7.3, and 7.8 $\mu\text{g PAB/g.}$, respectively, of dry yeast. The values obtained at low levels are in general somewhat higher than those found at intermediate levels. The same results were obtained with many other samples of yeast and incline us to the view that the values at intermediate levels are the most reliable. The recoveries obtained at different levels of added PAB range from 92 to 97%. The accuracy of the assay is $\pm 10\%$.

Specificity of Assay

Beadle and Tatum (12) tested a number of compounds structurally related to PAB and found that none of them had more than a fraction of a per cent of the activity of PAB. Thompson *et al.* (8) found that the acetyl derivative of the amide of PAB had 3% of the activity of PAB and attributed this activity to hydrolysis of the derivative by the mold rather than to possible contamination of the derivative with PAB. Several other investigators (13,14,15) have tested one or more such compounds for ability to counteract the inhibiting effect of sulfa

TABLE I
Reproducibility of the Assay

| Standard curve | | | Analysis of reference yeast | | | |
|------------------|---------------------|------------|-----------------------------|-------------------------|------------|------------|
| PAB per flask | Dry wt. of mycelium | | Sample | Dry wt. of mycelium PAB | | |
| | 5 runs | Average | | 5 runs | Average | Per g. |
| <i>mμg.</i> | <i>mg.</i> | <i>mg.</i> | <i>mg.</i> | <i>mg.</i> | <i>mg.</i> | <i>μg.</i> |
| 0 | 0-0.2 | 0.1 | 1 | 8.5-10.0 | 9.2 | 9.0 |
| 20 | 19.4-21.3 | 20.3 | 2 | — | 16.6 | 8.0 |
| 40 | 29.0-33.4 | 31.2 | 3 | 20.0-23.7 | 21.8 | 7.3 |
| 60 | 38.8-43.6 | 41.2 | 5 | 29.3-34.0 | 31.6 | 7.8 |
| 80 | 45.8-48.1 | 46.9 | | | | |
| 100 | 49.2-54.0 | 51.6 | | | | |

| Recovery of Added PAB | | | |
|----------------------------------|--------------------|-------------------|-------------------|
| Sample | Dry mycelium | PAB | |
| | | Found | Calculated |
| 1 mg. yeast + 20 <i>mμg.</i> PAB | <i>mg.</i> 25.5 | <i>mμg.</i> 28 | <i>mμg.</i> 29 |
| 2 mg. yeast + 20 <i>mμg.</i> PAB | 28.5 | 33 | 36 |

drugs on *Neurospora crassa* and bacteria and reported that some of the compounds contained the equivalent of from 0.1 to 6% free PAB. If such contamination were incident to the preparation of the compound it is to be expected that the percentage of impurity might vary with different lots. The figures that we have obtained show large variations among the compounds tested.

The following compounds, for which we are indebted to Dr. Brian L. Hutchings, Lederle Laboratories, were tested for growth-promoting activity: *p*-aminobenzoyl- β -alanine, *p*-aminobenzoyl-L-aspartic acid, *p*-aminobenzoyl-DL-aspartic acid, *p*-aminohippuric acid, *p*-aminobenzoylglutamic acid, *p*-aminobenzoyl- α -glutamyl- γ -glutamylglutamic acid tetraethyl ester (also the corresponding γ , α , and the α , α , compounds), *p*-nitrobenzoyl- α -glutamyl- γ -glutamic acid tetraethyl ester, *p*-nitrobenzoyl-L-amino-sebacic acid, *p*-nitrobenzoyl- γ -glutamyl- β -alanine, *p*-nitrobenzoylethanolamine, *p*-nitrobenzoyldiethanolamine, *p*-nitrobenzoyl-DL-alanine, *p*-nitrobenzoylpiperidine, *p*-nitrobenzoyl- α -glutamyl-DL-leucine, *p*-nitrobenzoyl- γ -glutamylglycine diethyl ester, *p*-nitrobenzoyl-DL- α -aminopimelic acid, *p*-nitrobenzoyl-DL- α -aminoadipic acid, *p*-nitrobenzoyl-DL-threonine, *p*-nitrobenzoylmor-pholine, *p*-nitrobenzoyl- α -glutamylglutamic acid, sodium pterate, pteroyl- γ -glutamyl- γ -glutamylglutamic acid, pteroylmonoglutamic acid

The compounds were used in molar proportions and were run at three levels corresponding to PAB values of 20, 60, and 100 $\mu\text{g.}/\text{flask}$. Most of the compounds had no activity, indicating the very specific nature of the assay. Only 9 compounds had an activity equivalent to 5% or more of that possessed by PAB. These were the first five amino compounds listed above and *p*-nitrobenzoyl-DL-threonine, *p*-nitrobenzoyldiethanolamine, and pteric acid. In order to ascertain if the compounds were free of PAB, three showing considerable activity, *viz.*, *p*-aminobenzoyl- β -alanine, *p*-aminobenzoyl-L-aspartic acid, and *p*-aminobenzoylglutamic acid were extracted with ether and the residues again assayed at the same levels as were used previously. All three compounds after extraction had no activity, indicating that they had been contaminated with PAB. The slight activity shown by the other compounds was probably due also to such contamination. Probably the contamination came about from the use of PAB in the synthesis of the derivatives. These results illustrate how unsafe it is to assume activity of a related compound unless it has been carefully purified. We were particularly conscious of possible PAB contamination because of having previously encountered such contamination from handling PAB in the same room with other materials (6). More recently we tried to use the *Cl. acetobutylicum* assay in a laboratory where other work with PAB was going on, but had to abandon it because the tubes could not be kept free of contamination from air-borne PAB.

Since none of the derivatives of PAB had any activity, it appears that whenever the PAB molecule is linked with an amino acid, the activity is lost, or, in other words, this organism responds only to free PAB. Among the various nitro compounds tested, only a few showed slight activity, which was also probably due to these nitro compounds

being slightly contaminated with PAB. Inactivity of nitro compounds is quite in agreement with the finding of Thompson *et al.* (8) that *p*-nitrobenzoic acid does not have any activity for this organism.

It is interesting that pteroylmono- and triglutamic acids were completely inactive for this mutant. Therefore, PAB must function in some other way than in the synthesis of pteroylglutamic acid, for the latter cannot replace PAB in the metabolism of this mold.

Comparison with the Chemical Method of Bratton and Marshall

Since this assay was to be used mostly to study PAB synthesis by yeasts, it was thought desirable to compare it with the chemical method of Bratton and Marshall. Figure 2 gives a comparison of the PAB values of the filtrate and the cells of five different strains of yeasts grown in the synthetic medium. It is evident from Fig. 2 that the PAB values of the filtrates obtained by the two methods are in good agreement. Since the test organism responds only to the free form of PAB, while the chemical method determines total diazotizable arylamines, and since there was no appreciable difference between the values obtained by the two methods, it is evident that only the free form of PAB must

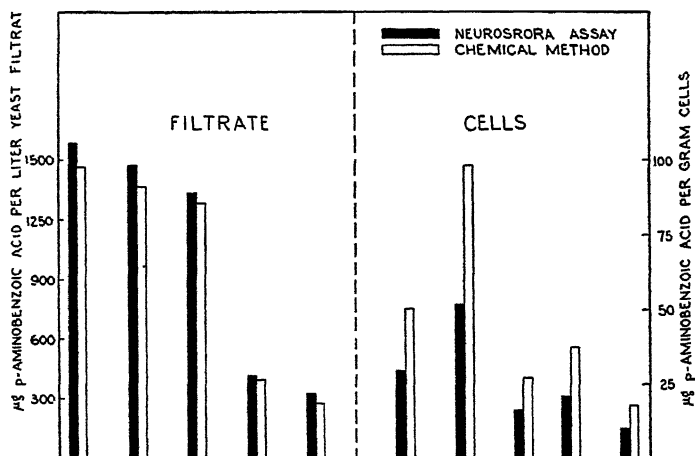


FIG. 2. Comparison of *Neurospora* assay with chemical method of Bratton and Marshall. A. *Saccharomyces cerevisiae* "Gebruder Mayer." B. *S. cerevisiae* Y-30. C. *S. cerevisiae* "Old Process." D. German press yeast, Lesser 105. E. *Candida albicans*.

be present in the fermented medium. This is not true for the cells, where the chemical method gave much higher values than the *Neurospora* assay. In one of the yeasts, *Saccharomyces cerevisiae* Y-30, the chemical method gave twice the value found by microbiological assay. These differences can presumably be ascribed to other diazotizable arylamines in the cells.

Extraction of PAB from Yeast Cells

To study the proportion of bound and free PAB in yeast cells, an experiment was set up in which the cells of five different yeasts³ were subjected to extraction by different reagents. The data are given in Table II. The amount of PAB extractable with water represents the

TABLE II
*Extraction of p-Aminobenzoic Acid from Yeast Cells
by Different Reagents^a*

μg. PAB/g. of cells extracted by

| | H ₂ O | 1 N H ₂ SO ₄ | 2 N H ₂ SO ₄ | 1 N HCl | 2 N HCl | 2 N NaOH | 5 N NaOH |
|----------------------------|------------------|---------------------------------------|---------------------------------------|------------|------------|-------------|-------------|
| <i>S. cerevisiae</i> Y-30 | 18.1 | 23.3 | 24.5 | 20.5 | 25.1 | 18.2 | 24.5 |
| <i>S. cerevisiae</i> G. M. | 17.7 | 17.7 | 22.1 | 18.0 | 25.9 | 13.2 | 18.5 |
| <i>T. utilis</i> 3 | 1.6 | 7.1 | 9.2 | 8.9 | 12.5 | 1.2 | 5.5 |
| <i>C. krusoides</i> 107 | 2.5 | 7.8 | 10.5 | 10.1 | 11.9 | 4.7 | 7.3 |
| <i>M. lipolytica</i> P-13 | 17.7 | 18.2 | 20.2 | 18.2 | 24.8 | 14.3 | 17.1 |

^a The yeast, 0.1 g., was autoclaved with 5 ml. of reagent at 15 lb. pressure for 1 hr.

free PAB in the cells, and the difference between this and the values obtained with the various hydrolyzing agents shows the amount of the bound form. Although the different yeasts varied in their content of free PAB, the bound form was almost the same in the yeasts tested. It varied only from 7.0 μg./g. for *Saccharomyces cerevisiae* Y-30 to 10.9 μg./g. for *Torula utilis*. Also *T. utilis*, which has the least amount of free PAB, had the maximum amount of bound PAB. Acid hydrolysis gave higher values than alkaline hydrolysis. Highest figures were obtained

³ In previous publications from this laboratory, the name *Candida arborea* was applied to one of these yeasts as per the label on the transfer at the time it was received. Because serious doubt as to the validity of the name *Candida arborea* exists among mycologists, a transfer was submitted to Dr. L. J. Wickerham of the Northern Regional Research Laboratories, who identified it as *Candida krusoides*, Castellani.

with the 2 *N* strength of the acid reagents. Three samples, *S. cerevisiae* G. M., *Mycotorula lipolytica* and *T. utilis*, gave a slight loss of PAB by 2 *N* NaOH treatment as compared with water extraction, but at 5 *N* NaOH strength, the liberation of PAB more than compensated for its destruction, and the values obtained are in general higher. It is not improbable that the increased PAB arose from the breakdown of such compounds as pteroylglutamic acid (PGA). More data on this question will be given in Table III.

Liberation of PAB from Liver Extract L

Figure 3 shows the liberation of PAB from Wilson's liver extract L, by acid and alkali at 15 lb. pressure. After hydrolysis all the samples

TABLE III

PAB Released on Hydrolysis of Pteroylglutamic Acid (PGA) by Different Reagents

| Sample | Reagent ^a | PAB found <i>μg./g.</i> | PAB from PGA <i>μg.</i> | PGA broken down <i>per cent</i> |
|--------------------------------------|---|----------------------------|-------------------------------|---------------------------------------|
| Reference yeast | H ₂ O | 8.4 | | |
| Reference yeast | 2 <i>N</i> NaOH | 12.5 | | |
| Reference yeast | 2 <i>N</i> H ₂ SO ₄ | 12.0 | | |
| Reference yeast + 100 <i>μg.</i> PGA | 2 <i>N</i> NaOH | 16.8 | 4.3 | 13.9 |
| Reference yeast + 100 <i>μg.</i> PGA | 2 <i>N</i> H ₂ SO ₄ | 22.2 | 10.2 | 32.9 |
| PGA, 100 <i>μg.</i> | 2 <i>N</i> NaOH | 4.8 | 4.8 | 15.5 |
| PGA 100 <i>μg.</i> | 2 <i>N</i> H ₂ SO ₄ | 12.0 | 12.0 | 38.8 |
| PGA 100 <i>μg.</i> | H ₂ O | <i>b</i> | <i>b</i> | <i>b</i> |

^a One-tenth g. of dry yeast, 100 *μg.* PGA, or combination of the two were autoclaved with 5 ml. of reagent for 1 hr. at 15 lb. pressure.

^b No weighable mycelial growth.

were brought to pH 5 before assaying. Extraction with water indicates a regular increase with time in the PAB content. The curves with H₂SO₄, HCl, and NaOH are typical and are almost the same as those obtained by Lampen and Peterson (6) with liver A. With acids, an approximately 50-fold increase in PAB was obtained in 1 hr. Following this, there was a continuous loss, which may be attributed to either destruction of PAB or conversion into a form unavailable to the organism.

Alkaline hydrolysis with 2 *N* NaOH released PAB more slowly, but liberation continued longer than with acids of the same strength. The

values reached at the end of 8 hr. were much higher with alkaline hydrolysis. The 5 *N* NaOH curve was regularly higher than the 2 *N* curve, but neither one reached a plateau even after 8 hr. A similar experience is reported by Lampen and Peterson (6) who obtained maximal values only after autoclaving with 5 *N* NaOH at 75–80 lb. pressure for 1 hr. A run made with undefatted whole liver gave almost identical curves, although less PAB/g. was obtained.

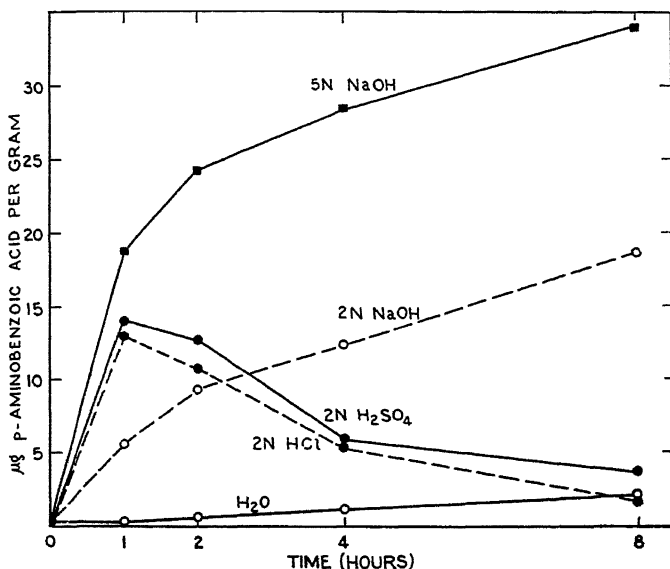


FIG. 3. Liberation of *p*-aminobenzoic acid from liver extract L by various reagents, at 120°C.

Since there is always an increase in PAB content of natural materials by alkaline or acid hydrolysis, it is tempting to use these methods for PAB extraction. However, if the increased PAB comes from PGA it would seem undesirable to use such methods because folic acid is measured in other ways. Table III shows the effect of various hydrolyzing agents on production of PAB from folic acid (PGA) under various conditions. It is apparent that these procedures convert some but not all of the PGA into free PAB. The PGA content of yeast (*S. cerevisiae*) is said to range from 20 to 35 $\mu\text{g./g.}$ dry basis (16,17). Assuming one-third

hydrolysis by acid, the PGA could give from 2.3 to 4.0 $\mu\text{g.}$ of PAB which is about the increase that was obtained on hydrolysis of the yeast (3.6 $\mu\text{g.}$). The PAB thus obtained should obviously not be included in the PAB values. Hence, even though minimal values are obtained with water extraction, it appears preferable to acid or alkaline hydrolysis.

SUMMARY

An improvement in the assay of *p*-aminobenzoic acid (PAB) with *p*-aminobenzoicless strain *Neurospora crassa* 1633 has been obtained by supplementing the basal medium with asparagine and casein hydrolysate. This medium gives optimal growth and extends the range of the assay to 100 $\mu\text{g.}/25$ ml. medium. PAB recoveries of 92–97% were obtained at the different levels.

A large number of compounds related to PAB have been tested and most of these were inactive. Three compounds that seemed to have appreciable activity proved to be inactive after extraction with ether. From these results it is concluded that these compounds were contaminated with free PAB. *Neurospora crassa* 1633 apparently responds only to free PAB. Pteroylglutamic acid (PGA), for example, was completely inactive.

Good agreement between the *Neurospora* assay and the chemical method of Bratton and Marshall was obtained for yeast filtrates but higher values were given by the chemical method for yeast cells. These data indicate that the filtrates contain only free PAB whereas the cells contain other diazotizable arylamines as well as PAB.

PAB in yeast cells occurs in both free and bound form. The best hydrolyzing agent for the bound PAB was 2 *N* HCl. It is questionable whether PAB values obtained in this way should be included in assay figures as much, if not all, of the increased PAB comes from hydrolysis of PGA and should be expressed as that vitamin rather than as PAB.

The PAB in Wilson's liver extract L was rapidly destroyed or converted to an unavailable form during acid hydrolysis. Autoclaving with 5 *N* NaOH gave the maximum PAB liberation, and the amount of PAB/g. continuously increased with the time of hydrolysis. The increased PAB probably arises from PGA, as this compound is readily split by alkali.

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Production of *p*-Aminobenzoic Acid by Representative Yeasts¹

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INTRODUCTION

The occurrence of *p*-aminobenzoic acid (PAB) in yeast has been known for a number of years and a considerable number of papers (1-6) have been published dealing with the amount and forms of the compound that are present. Most of these papers have been concerned with the PAB content of the yeast cell, whereas in fact this comprises but a small proportion of the total synthesized. Most of the PAB elaborated by the yeast cell is secreted into the surrounding medium and only a small part is retained within the cell. Lampen, Baldwin, and Peterson (7) reported a synthesis of 1800 $\mu\text{g./l.}$ *Saccharomyces cerevisiae*, and found 80% or more was in the medium and about 20% or less remained in the yeast cells. They also studied the optimum methods of extraction of free PAB from the yeast cells and found that most of it could be extracted simply by autoclaving the cells with water for 1 hr. at 15 lb. pressure. The purpose of the present study was to extend the investigation of Lampen *et al.* to a considerable number of representative yeasts and to determine the amount of PAB synthesized by such yeasts in both natural and synthetic media.

EXPERIMENTAL

Yeast Strains

Transplants of *Candida albicans*, *Endomyces magnusii* 836, *Hansenula anomala* var. *spherica* 778, *Saccharomyces cerevisiae* 53, *S. cerevisiae* Y-30, *S. cerevisiae* "Gebruder Mayer," *S. cerevisiae* "Old Process," *Torula utilis* 1, *T. utilis* 2, *T. utilis* 3, *Torula*

¹ Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

² Government of India Scholar.

cremoris, *Willia anomala*, and *Mycotorula lipolytica* P-13 were kindly supplied by Dr. Elizabeth McCoy of the Department of Agricultural Bacteriology. Cultures of *Candida krusoides* and *Candida pulcherrima* were sent to us through the courtesy of Mr. H. J. Bunker, Barkley Perkins and Co., Ltd., London, and Dr. K. R. Butlin, Department of Scientific and Industrial Research, Chemical Research Laboratory, Teddington. Slants of *Hansenula saturnas*, *Endomycopsis fibuliger*, *Mycoderma cerevisiae*, *Candida zeylanoides*, *Candida krusei*, *Candida guilliermondii*, *Pichia alcoholophila*, *Debaryomyces globosus*, *Debaryomyces matrucoti* were kindly given to us by Dr. L. J. Wickerham of the Northern Regional Research Laboratory, Peoria. For a culture of *Rhodotorula gracilis* we are indebted to Dr. Harry Lundin, Kungl. Tekniska Hogskolan, Stockholm. In former papers from this laboratory *Candida krusoides* was called *Candida arborea* as per the label on the transplant when it was received. Dr. Wickerham has examined the culture and identified it as *Candida krusoides*, Castellani.

All yeasts were maintained on agar slabs containing 0.25% glucose, 0.25% Difco yeast extract and 1.5% Bacto agar. Transfers were made at monthly intervals.

Media and Fermentation

The inoculum medium contained 2.0% beet molasses, 0.75% corn steep liquor, and 0.1% $(\text{NH}_4)_2\text{HPO}_4$. A sterilized solution of ammonium phosphate was added to the clarified and sterilized solution of molasses, corn steep liquor, and malt extract just before inoculation. The fermentation medium containing clarified Ovid Colorado No. 1 molasses was prepared according to the method of Agarwal and Singh (8). The medium was prepared so as to contain about 1% sugar calculated as sucrose, 2.0% of the previously treated corn steep liquor and 0.1% of $(\text{NH}_4)_2\text{HPO}_4$. For clarification, the crude corn steep liquor was diluted three times, steamed for 30 min., cooled, and filtered.

The synthetic medium for inoculum and fermentation was the same as that used by Olson and Johnson (9).

The inoculum was grown for 18 hr. at 30°C. in 500-ml. Erlenmeyer flasks, containing 20 ml. of medium, on a rotary shaker (made by B. F. Gump Co., Chicago) describing a 2.25-in. circle at 250 r.p.m. The fermentations were run with 50 ml. medium/flask for 24 hr. at 30°C. on the same shaker. A 5% inoculum was used to start the fermentation.

After fermentation the cells were separated from the medium by centrifugation at 2000 r.p.m. for 5 min. and washed twice with 25 ml. of distilled water. The supernatant and the washings were combined. The washed cells were suspended in 50 ml. of distilled water and aliquots were taken for dry weight analysis.

Analytical Methods

The dry weight of yeast was determined in duplicate by centrifuging 5 ml. of the homogeneous suspension at 2000 r.p.m. for 5 min. in weighed Pyrex test tubes, 15 × 125 mm., drying at 109°C. for 16–18 hr. and weighing. The yield of dry yeast was expressed as per cent of sugar fermented.

Sugar determinations after hydrolysis were made according to the micro method of Shaffer and Somogyi (10), with reagent No. 50 containing 5 g. of KI/l.

PAB was determined according to an improved method (11) by means of the "*p*-aminobenzoicless" strain of *Neurospora crassa* No. 1633, produced by Tatum and Beadle (12). For assaying the vitamin in the cells, 10 ml. of the above washed homogeneous suspension was autoclaved with the same volume of water at 15 lb. pressure for 1 hr., and aliquots used. For PAB values of the supernatant, the fermented medium and the washings were combined, proper dilution was made, and aliquots were used for assay. A reference sample of pulverized yeast assaying 8.5–9.0 $\mu\text{g./g.}$ was always included with each assay, as an additional check on the method.

RESULTS AND DISCUSSION

Synthesis of PAB in Molasses and Corn Steep Liquor Medium

Table I shows the yields obtained and PAB synthesized, in natural medium, by 25 strains of yeasts representing 12 genera. In general, high yields of the yeast cells based on the sugar fermented were obtained. Three strains—*C. zeylanoides*, *D. matruchoti*, and *R. gracilis* gave over 70% yields. All the other strains tested gave yields ranging from 50 to 70% except 6 strains which gave yields below 50%. Probably the high yields obtained were due to utilization of non-sugar carbon as reported for other yeasts by Agarwal and Peterson (13). The low yields of *T. cremoris* (20.4%) and *W. anomala* (13.1%) may be related to the conversion of sugar to other products, *e.g.*, alcohol and acids, rather than to cells. Even with these organisms, however, sugar utilization was complete.

It is apparent from the table that all strains of yeasts tested synthesized PAB during growth, but the amount varied with the strain. The highest producer of PAB was *M. lipolytica*, which synthesized 8470 $\mu\text{g./l.}$, while the lowest amount of PAB, 160 $\mu\text{g./l.}$, was synthesized by the widely used strain *T. utilis* No. 3. Since the yields of *T. cremoris* and *W. anomala* were low, much less PAB per liter was synthesized. It is interesting to note that with the exception of *W. anomala*, the same yeasts *M. lipolytica*, *D. globosus*, *S. cerevisiae* "Old Process" and *M. cerevisiae*, that produced the highest amount of PAB per liter also synthesized the highest amount of PAB on a per gram basis. Thus with the exception noted, there is a very good correlation between the total synthesis and the synthesis per gram of cells. The same relationship holds for the organisms producing the lower amount of PAB.

A variation in PAB synthesis among strains of the same species and among species of the same genus may also be noted from the data given in Table I. Thus the strain "Old Process" of *S. cerevisiae* synthesized 680 $\mu\text{g./g.}$ of PAB, while 340 $\mu\text{g./g.}$ was produced by the "Gebruder

TABLE I
Synthesis of PAB by Certain Yeasts in Molasses Medium

| Yeast | Yield of dry yeast | | PAB content | | Total PAB synthesis ^a | |
|----------------------------|--------------------------|--------|-------------------------------|----------|----------------------------------|--------------|
| | Based on sugar fermented | Weight | Of fermented cell free medium | Of cells | | |
| | % | g./l. | μg./l. | μg./g. | μg./l. | μg./g. cells |
| <i>C. albicans</i> | 67.8 | 7.12 | 940 | 33.3 | 1130 | 160 |
| <i>C. krusoides</i> | 64.4 | 6.58 | 1450 | 20.7 | 1540 | 230 |
| <i>C. pulcherrima</i> | 54.7 | 5.64 | 820 | 1.8 | 770 | 140 |
| <i>C. zeylanoides</i> | 74.8 | 6.14 | 970 | 16.9 | 1030 | 170 |
| <i>C. krusei</i> | 58.6 | 4.82 | 220 | 3.8 | 200 | 40 |
| <i>C. guilliermondii</i> | 69.1 | 6.70 | 2000 | 11.6 | 2030 | 300 |
| <i>D. globosus</i> | 36.8 | 3.36 | 3050 | 33.6 | 3120 | 930 |
| <i>D. matruchoti</i> | 79.6 | 7.56 | 1270 | 2.8 | 1240 | 160 |
| <i>E. magnusii</i> | 67.1 | 6.78 | 600 | 8.1 | 610 | 90 |
| <i>E. fibuliger</i> | 37.5 | 4.10 | 610 | 9.7 | 610 | 150 |
| <i>H. anomala</i> | 58.9 | 5.84 | 350 | 3.4 | 340 | 60 |
| <i>H. saturnas</i> | 49.5 | 5.00 | 670 | 7.3 | 660 | 130 |
| <i>M. cerevisiae</i> | 54.9 | 4.54 | 2960 | 10.3 | 2960 | 650 |
| <i>M. lipolytica</i> P-13 | 69.8 | 6.46 | 8500 | 4.2 | 8470 | 1310 |
| <i>P. alcoholophila</i> | 66.3 | 5.44 | 2190 | 8.7 | 2200 | 400 |
| <i>R. gracilis</i> | 71.1 | 6.40 | 220 | 2.0 | 210 | 33 |
| <i>S. cerevisiae</i> 53 | 52.1 | 5.02 | 2090 | 2.5 | 2050 | 410 |
| <i>S. cerevisiae</i> Y-30 | 51.7 | 4.78 | 1750 | 4.8 | 1750 | 370 |
| <i>S. cerevisiae</i> G. M. | 54.3 | 5.48 | 1650 | 47.4 | 1870 | 340 |
| <i>S. cerevisiae</i> O. P. | 44.8 | 4.50 | 2810 | 62.2 | 3050 | 680 |
| <i>T. cremoris</i> | 20.4 | 2.14 | 1170 | 19.7 | 1180 | 550 |
| <i>T. utilis</i> 1 | 69.8 | 6.28 | 470 | 0.6 | 420 | 67 |
| <i>T. utilis</i> 2 | 62.3 | 6.58 | 270 | 1.5 | 230 | 34 |
| <i>T. utilis</i> 3 | 54.4 | 5.76 | 200 | 1.9 | 160 | 27 |
| <i>W. anomala</i> | 13.1 | 1.38 | 2050 | 13.4 | 2030 | 1470 |

^a The PAB content of the unfermented medium ranged from 23 to 54 μg./l. depending upon the batch of corn steep liquor and also upon the slight variation in the quantity used. This has been deducted in the calculation and the figures expressed to nearest ten, e.g., for *C. albicans*, $7.12 \times 33.3 + 940 - 54 = 1130$. The figures in the last column are obtained by dividing those in the preceding column by the values in Col. 3, thus $1130 \div 7.12 = 160$.

Mayer" strain. *C. guilliermondii* produced 300 μg./g. of PAB, while *C. krusei* synthesized only 40 μg./g.

Since among the strains tested *M. lipolytica* produced the greatest amount of PAB, it was thought that PAB might be associated with fat

production. Another fat-producing yeast, *R. gracilis*, was therefore tested for its PAB production. However, this organism synthesized only 33 μ g.PAB/g. compared with the 1310 μ g./g. produced by *M. lipolytica*. PAB synthesis does not appear to be related to fat production.

The data of Table I also show that all yeasts secrete about 90–99% of the PAB synthesized into the medium. An exception to this generalization was noted with *C. albicans* which secreted only 79% into the medium. These results are in agreement with the work of Lampen, Baldwin, and Peterson (7), who noted the same results with bakers' yeast. The high proportion of the total PAB found in the cell-free medium is similar to that observed with many other B-vitamins. If these are synthesized by the cell, most of the product, as a rule, is secreted into the surrounding medium instead of being retained in the cell (14–18).

The PAB content of the cells was different for each species and strain studied. From the data in Table I, no correlation could be found between the total amount of the PAB produced and the amount retained in the cells. However, later work with a few strains of yeast indicates that the amount of PAB remaining in the cells is dependent upon the age of the cells and the external conditions of the medium.

Synthesis of PAB in Synthetic Medium

To supplement the observation of the synthesis of PAB in the molasses medium, some yeasts were grown in the synthetic medium and their PAB production studied. The results obtained have been presented in Table II. The sugar fermented in most cases was 98% or more. In general the yields obtained here were lower than those in molasses medium, except for *E. fibuliger*, which gave a slightly higher yield of 44.7% in the synthetic as compared with 37.5% in molasses medium. *C. pulcherrima* and *W. anomala* did not show any growth in 24 hr., probably because they require some growth factor not present in this medium.

Since cell yields were lower, less PAB per liter was produced by the yeasts in this medium. Most of the organisms produced less PAB per gram of dry cells (Col. 7) than in the natural medium. A maximum difference was observed with *E. fibuliger*, which synthesized only 1/12 as much PAB in the synthetic medium as in the molasses medium. A reduction greater than 50% was observed with *M. lipolytica* and *C.*

guilliermondii. A possible explanation for this phenomenon may be the presence of some factor in molasses or corn steep liquor which favors PAB synthesis. However, about $\frac{1}{3}$ of the yeasts gave a higher PAB synthesis per gram of cells in the synthetic medium than in the molasses medium; e.g., strains of *S. cerevisiae* Y-30 and "Old Process." To determine the validity of these differences between the PAB synthesis in the two kinds of medium, runs were made to test the consistency of PAB production under the same conditions. Duplicates of different yeasts

TABLE II
Synthesis of PAB by Certain Yeasts in Synthetic Medium^a

| Yeast | Yield of dry yeast | | PAB content | | Total PAB synthesis | |
|----------------------------|--------------------------|--------|-------------------------------|----------|---------------------|--------------|
| | Based on sugar fermented | Weight | Of fermented cell free medium | Of cells | | |
| | % | g./l. | μg./l. | μg./g. | μg./l. | μg./g. cells |
| <i>C. albicans</i> | 52.9 | 5.54 | 460 | 12.8 | 530 | 96 |
| <i>C. krusoides</i> | 36.9 | 3.76 | 90 | 4.8 | 110 | 29 |
| <i>C. zeylanoides</i> | 20.3 | 1.46 | 110 | 1.0 | 110 | 74 |
| <i>C. krusei</i> | 26.3 | 2.86 | 120 | 0.8 | 120 | 42 |
| <i>C. guilliermondii</i> | 28.5 | 3.12 | 370 | 0.8 | 370 | 120 |
| <i>D. globosus</i> | 13.2 | 1.42 | 570 | 0.8 | 570 | 400 |
| <i>D. matruchoti</i> | 38.1 | 4.18 | 600 | 1.1 | 800 | 190 |
| <i>E. magnusii</i> | 25.6 | 1.56 | 500 | 1.6 | 500 | 320 |
| <i>E. fibuliger</i> | 44.7 | 4.74 | 62 | 0.3 | 63 | 13 |
| <i>H. anomala</i> | 46.1 | 4.76 | 80 | 4.8 | 100 | 21 |
| <i>H. saturnus</i> | 26.3 | 2.88 | 400 | 1.2 | 400 | 142 |
| <i>M. cerevisiae</i> | 31.4 | 2.48 | 1800 | 0.7 | 1800 | 730 |
| <i>M. lipolytica</i> P-13 | 32.4 | 3.82 | 2400 | 5.4 | 2420 | 630 |
| <i>P. alcohophilus</i> | 19.1 | 1.50 | 660 | 1.0 | 660 | 440 |
| <i>R. gracilis</i> | 34.7 | 4.16 | 50 | 3.0 | 62 | 15 |
| <i>S. cerevisiae</i> 53 | 19.0 | 1.96 | 400 | 2.6 | 400 | 200 |
| <i>S. cerevisiae</i> Y-30 | 23.8 | 2.86 | 1720 | 62.9 | 1900 | 660 |
| <i>S. cerevisiae</i> G. M. | 28.9 | 2.98 | 1610 | 15.1 | 1650 | 550 |
| <i>S. cerevisiae</i> O. P. | 25.1 | 2.52 | 2030 | 4.8 | 2040 | 810 |
| <i>T. cremoris</i> | 13.0 | 1.32 | 610 | 6.7 | 620 | 470 |
| <i>T. utilis</i> 1 | 28.3 | 2.60 | 140 | 11.9 | 170 | 66 |
| <i>T. utilis</i> 2 | 36.3 | 3.96 | 50 | 4.5 | 70 | 18 |
| <i>T. utilis</i> 3 | 50.8 | 4.06 | 20 | 4.7 | 40 | 10 |

^a PAB content of the unfermented medium 0 μg./l. For calculation of total PAB see footnote to Table I.

were grown for 24 hr. in synthetic medium containing 1% sugar and the results obtained are presented in Table III. The yields of cells as represented by Col. 3 were quite consistent for the yeasts tested. PAB synthesis on a per gram basis did not vary more than about 10% from the average for individual runs except in the case of *T. utilis*. The synthesis here was so small that great variations might be expected. On a per liter basis, the syntheses show marked variations among duplicates, as would be expected because of variations in yields. By and large, pro-

TABLE III
Reproducibility of PAB Synthesis by Yeasts^a

| Yeast | Run | Dry cells | Yield on fermented sugar | PAB content | | Total PAB synthesis | |
|---------------------------|-----|-----------|--------------------------|-------------------------------|----------|---------------------|--------------|
| | | | | Of fermented cell free medium | Of cells | | |
| | | g. l. | % | μg./l. | μg./g. | μg./l. | μg./g. cells |
| <i>C. kruseoides</i> | 1 | 3.90 | 34.8 | 115 | 5.1 | 135 | 35 |
| <i>C. kruseoides</i> | 2 | 4.08 | 35.2 | 100 | 10.1 | 141 | 34 |
| <i>M. lipolytica</i> P-13 | 1 | 5.52 | 50.6 | 3250 | 10.7 | 3310 | 600 |
| <i>M. lipolytica</i> P-13 | 2 | 4.74 | 43.0 | 2300 | 11.8 | 2356 | 708 |
| <i>S. cerevisiae</i> Y-30 | 1 | 4.40 | 40.0 | 2380 | 66.0 | 2670 | 607 |
| <i>S. cerevisiae</i> Y-30 | 2 | 3.52 | 30.0 | 1760 | 55.4 | 1955 | 556 |
| <i>T. utilis</i> 3 | 1 | 5.18 | 43.9 | 9.4 | 1.6 | 17.7 | 3.4 |
| <i>T. utilis</i> 3 | 2 | 5.80 | 49.8 | 2.2 | 1.4 | 10.3 | 1.8 |

^a All the fermentations were run in synthetic medium containing 1% sugar for 24 hr.

duction of PAB by a given yeast is tied to the yield of the cells. The cells of *S. cerevisiae* Y-30 contained the most PAB (55.4–66.0 μg./g.) and the least amount was found in *T. utilis* 3 (1.4–1.6 μg.).

The last columns of Tables I, II and III point to a rough generalization, i.e., variation in PAB synthesis among species of a genus is much less than the difference among yeasts that do not belong to the same genus. Thus *Saccharomyces* may be regarded as high, *Torula* as low, and *Candida* (with one exception) as moderate producers.

Synthesis of PAB under Anaerobic Conditions

To study the effect of aeration on the production of PAB, 10 yeasts were grown in molasses plus corn steep liquor medium, under strict and partial anaerobiosis. The inoculum was grown aerobically, and 5% was used to start the fermentation. The fermentations were run in tubes of 32×200 mm. containing 50 ml. medium. For partial anaerobiosis the tubes were simply placed in the incubator or kept at 30°C . for 72 hr. For strict anaerobiosis, the tubes were in an anaerobic oat jar during incubation. Table IV gives the yield of cells and PAB synthesized by

TABLE IV
Comparison of the PAB Synthesis by Some Yeasts Under Aerobic
and Anaerobic Conditions (Molasses Medium)

| Yeast | Yield of dry cells, per cent of sugar fermented | | | PAB synthesized, $\mu\text{g./g.}$ dry cells | | |
|----------------------------|--|---------------------|-------------------------|--|---------------------|-------------------------|
| | Aerated medium | Unaerated medium | Anaerobic conditions | Aerated medium | Unaerated medium | Anaerobic conditions |
| <i>C. albicans</i> | 67.8 | 9.2 | 8.1 | 160 | 168 | 130 |
| <i>C. krusoides</i> | 64.4 | 3.6 | 3.4 | 230 | 196 | 130 |
| <i>E. magnusii</i> | 67.1 | 21.1 | 2.1 | 90 | 293 | 300 |
| <i>M. lipolytica</i> | 69.8 | 3.7 | 1.9 | 1310 | 2174 | 2380 |
| <i>S. cerevisiae</i> 53 | 52.1 | 7.8 | 5.0 | 410 | 196 | 170 |
| <i>S. cerevisiae</i> G. M. | 54.3 | 9.5 | 7.9 | 340 | 446 | 110 |
| <i>S. cerevisiae</i> O. P. | 44.8 | 9.1 | 7.7 | 680 | 427 | 100 |
| <i>T. cremoris</i> | 20.4 | 6.7 | 6.5 | 550 | 261 | 230 |
| <i>T. utilis</i> 3 | 54.4 | 8.7 | 3.4 | 27 | 181 | 240 |
| <i>W. anomala</i> | 13.1 | 7.6 | 5.9 | 1470 | 840 | 210 |

the different strains under partial anaerobiosis and in the oat jar experiment. It is apparent from the table that the yield of cells under partial anaerobiosis (unaerated) were somewhat higher than under completely anaerobic conditions. The maximum variation was shown by *E. magnusii*, which gave yields of 21.2% and 2.12% respectively. The least difference was observed with *C. krusoides*, which gave almost the same yields under both conditions. *M. lipolytica* gave almost twice as much yeast when air was allowed to diffuse into the medium as when it was rigidly excluded.

Comparison of the PAB synthesis under aerobic, partially anaerobic and strict anaerobic conditions shows that on a per gram of cells basis,

5 of the 10 strains produced most PAB under aerobic conditions, less under partial anaerobiosis, and least under strictly anaerobic conditions. This is particularly striking with *W. anomala*, which produced 1470 $\mu\text{g. 'g.}$, 840 $\mu\text{g. 'g.}$, and 210 $\mu\text{g. 'g.}$, as conditions were varied from aerobic to strictly anaerobic. The reverse effect was obtained with *M. lipolytica*, *T. utilis*, and *E. magnusii*. These yeasts synthesized maximum PAB under strict anaerobiosis, and least under aerobic conditions. *C. albicans* and *S. cerevisiae* G.M. produced more PAB aerobically than anaerobically, but were not so sensitive as the other organisms to variations in the conditions.

If the synthesis is calculated on a per liter of medium basis instead of on a per gram of cells basis, the amount of PAB synthesized under aerobic conditions is equal to or several times that produced under partial or strict anaerobiosis. The yield of cells is so much greater under aerobic conditions that even though the synthesis per gram is less, the over-all is greater than that obtained under anaerobic conditions.

The distribution of PAB between the cells and the medium under these conditions was about the same as under aerobic conditions (Table I). From 93 to 99% of the PAB synthesized was found in the medium.

Nature of the PAB Secreted into the Medium

Since the yeasts were capable of producing PAB in both natural and synthetic medium and 90–99% or more was secreted into the medium, it was thought advisable to study the nature of the secreted PAB. With this object in view a distribution experiment was set up to determine if the compound in the medium had the properties of free PAB. Ten ml. of the yeast filtrate was adjusted to different pH values and extracted with an equal volume of ether or *n*-butanol. The extracted aqueous layer was readjusted to pH 5 and aliquots were assayed. Table V gives the data on extraction of PAB and an active substance from yeast cultures by ether and butanol at different pH levels. Much better extraction of PAB and of the active substance in the yeast filtrate was obtained with ether at pH 4.5 than at pH 8.5. At the upper level, only 2.5% of the activity could be extracted, as compared with about 68% at pH 4.5. Similar results were obtained with butanol, with which about 90% of the activity was extracted at pH 3.5 and only about 25% at pH 7.0. It is apparent that the distribution of free PAB and the active substance of the yeast filtrates between solvents and water at different

TABLE V
Distribution Coefficients of Free PAB and PAB in Yeast Filtrates

| Solvent | pH of the aqueous phase | Sample, PAB or culture | Amount of PAB in | | | Activity extracted by solvent |
|---------------------------------------|-------------------------|------------------------|-------------------|---------------|--------------------------|-------------------------------|
| | | | Original solution | Aqueous phase | Solvent phase (by diff.) | |
| Ether (1:1 vol. ratio) | 4.5 | PAB | 2500 | 800 | 1700 | 68 |
| | | <i>S. cerevisiae</i> | 1520 | 470 | 1050 | 70 |
| | | <i>M. lipolytica</i> | 1640 | 600 | 1040 | 64 |
| | 5.5 | PAB | 2500 | 2430 | 70 | 2.8 |
| | | <i>S. cerevisiae</i> | 1520 | 1480 | 40 | 2.6 |
| | | <i>M. lipolytica</i> | 1640 | 1600 | 40 | 2.4 |
| <i>n</i> -Butanol (1:1 vol. ratio) | 3.5 | PAB | 2500 | 160 | 2340 | 93 |
| | | <i>S. cerevisiae</i> | 1520 | 130 | 1390 | 91 |
| | | <i>M. lipolytica</i> | 1640 | 180 | 1460 | 88 |
| | 7.0 | PAB | 2500 | 1910 | 590 | 24 |
| | | <i>S. cerevisiae</i> | 1520 | 1200 | 320 | 21 |
| | | <i>M. lipolytica</i> | 1640 | 1180 | 460 | 28 |

pH levels is the same, within experimental error. From these data it may be concluded that the PAB synthesized and secreted by *S. cerevisiae* and *M. lipolytica* is present in the free form. These data are in agreement with other results (11) that show the marked specificity of the test organism, *N. crassa*, for PAB.

SUMMARY

Representative yeasts of 12 genera were grown in both synthetic and natural medium, and were found to synthesize large quantities of PAB during growth. In molasses medium, the range of PAB synthesis varied from 27 $\mu\text{g.}/\text{g.}$ for *T. utilis* to 1310 $\mu\text{g.}/\text{g.}$ for *M. lipolytica*. In synthetic medium, the same yeasts gave respective yields of 10 $\mu\text{g.}$ and 630 $\mu\text{g.}/\text{g.}$ Hence molasses medium appears to contain some nutrient that favors PAB production. From 79 to 99% of the PAB produced was secreted into the medium surrounding the cell.

Under anaerobic conditions, the total PAB synthesis, i.e., per liter of medium, is less than that under aerobic conditions because PAB

production by and large is related to the yield of cells. On a per gram basis some yeasts, *e.g.*, *M. lipolytica*, showed greater synthesis under anaerobic conditions than under aerobic, but the reverse was true for most of the yeasts.

Since the distribution of PAB and the active material in the yeast filtrate of *S. cerevisiae* and *M. lipolytica* in ether and butanol at different pH levels was the same, the PAB secreted by these yeasts is believed to be of the free form.

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Effect of Changes in Medium on *p*-Aminobenzoic Acid Synthesis by Certain Yeasts¹

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INTRODUCTION

There have been very few reports on factors that affect the production of *p*-aminobenzoic acid (PAB) by yeasts. PAB synthesis by *Torula utilis* was adversely affected by a deficiency of iron according to Lewis (1). The rate of synthesis was reduced from 60 $\mu\text{g./g.}$ of cells for normal yeast to about 3 $\mu\text{g./g.}$ for yeast suffering from severe iron deficiency. The effect of delayed separation of the cells from the medium on the PAB content of *Torula utilis* was also studied by Lewis *et al.* (2), who reported a decrease from 18.3 $\mu\text{g./g.}$ (immediate separation) to 12.0 $\mu\text{g./g.}$ (delayed separation). In cultures refrigerated for longer periods of time as little as 2.05 $\mu\text{g./g.}$ was retained in the cells. A decrease in the amount of PAB formed by *Saccharomyces cerevisiae*, by the addition of thiamine, pyridoxine, alanine, and inositol was noted by Eppright and Williams (3). Addition of vitamins decreased the PAB from 42 $\mu\text{g./l.}$ to 0.83 $\mu\text{g./l.}$ Hartelius and Roholt (4) found young growing yeast to be more resistant to sulfanilamide than older cells and attributed this to the greater PAB content of the younger cells. They also found that the inhibitory effect of sulfanilamide on yeast was abolished after a long period of fermentation and explained it on the basis of PAB synthesis during the long period of growth. In a previous paper synthesis of PAB was reported (5) for 25 yeasts comprising 12 genera. The factor having the greatest effect on the synthesis of the vitamin appeared to be the type of yeast. A second factor affecting PAB production was aeration. In unaerated medium some yeasts produced less

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PAB per gram of cells and others produced more. Under strictly anaerobic conditions, reduced production was still more marked. However, if compared on a per volume of medium basis, PAB production was many times greater in the aerated medium. This is to be expected since in an aerated medium, cell production is also much increased. By and large PAB production parallels cell yields.

In the present investigation the effect of modifications in the culture medium on the extent of growth, on the synthesis of PAB, and on the amount of PAB retained in the cells has been studied. The kinetics of PAB synthesis of some yeasts has also been investigated.

EXPERIMENTAL

Yeast Strains

The following strains of yeasts were used for most parts of this study: *Saccharomyces cerevisiae* Y-30, *S. cerevisiae* "Gebruder Mayer," *S. cerevisiae* "Old Process," *Myotorula lipolytica*, P-13, *Torula utilis* 1, *T. utilis* 2, *T. utilis* 3, *Hansenula anomala* var. *spherica* 778, *Candida krusoides*, and *Rhodotorula gracilis*. The source of these yeasts has been given in a previous paper (5).

Media and Fermentation

The synthetic medium for inoculum and fermentation was that of Olson and Johnson (6). Fifty ml. in a 500-ml. Erlenmeyer flask was shaken at 200 r.p.m. during the fermentation period of 24 hr. Details regarding preparation of the inoculum and other procedures have been described previously (5).

Analyses

Yeast growth was measured by determining the dry weight of cells (5). Reducing sugar was determined by the micro method of Shaffer and Somogyi (7) (reagent No. 50 with 5 g. KI/l.), and calculated as glucose. PAB assays were run according to a modified method (8) with "p-aminobenzoicless" strain of *Neurospora crassa* No. 1633.

Kinetics of PAB Synthesis

In the previous paper (5), 90-99% of the PAB produced by all yeasts was found in the medium; an experiment was therefore set up to determine the rate of production. Three of the high producers, *Saccharomyces cerevisiae* Y-30, *S. cerevisiae* "Gebruder Mayer" and *Myotorula lipolytica* P-13, and two of the low producers *Hansenula anomala* 778 and *Candida krusoides*, were selected for this purpose. The PAB content of the cells and the fermented medium was determined every 4 hr. The addition of PAB through the inoculum was avoided by using a

washed suspension of the yeast cells. In Table I are presented the dry weight of cells, the sugar used, and the PAB produced by these yeasts during the successive periods. With *S. cerevisiae* sugar utilization was complete in 12 hr., but the weight of cells increased even after this time. This was probably due to the utilization of alcohol and other non-sugar carbon as reported for other yeasts by Agarwal and Peterson (9). Even though no sugar was present at the end of 12 hr., PAB was continuously synthesized and secreted into the medium. Production of PAB thus cannot be related directly to the breakdown of sugar, but appears to be dependent upon the growth of the cells. Data for the other three yeasts

TABLE I
Effect of Age on the Synthesis of PAB by Some Yeasts in Synthetic Medium^a

| Fermentation | Dry cells | Glucose used | PAB content | | Total PAB synthesis | Average PAB synthesis g, hr. at each interval |
|---------------------------------------|-----------|--------------|-------------------------------------|----------|---------------------|---|
| | | | Of fermented cell-free medium | Of cells | | |
| <i>S. cerevisiae</i> Y-30 | | | | | | |
| hr. | g./l. | g./l. | μg./l. | μg./g. | μg./g. | μg. |
| 4 | 0.28 | 0.90 | 24 | 6.0 | 89 | 34.2 |
| 8 | 0.75 | 4.14 | 76 | 7.0 | 108 | 26.7 |
| 12 | 2.00 | 11.08 | 260 | 18.2 | 148 | 39.1 |
| 16 | 2.70 | 11.08 | 830 | 24.1 | 331 | 63.6 |
| 24 | 3.50 | 11.08 | 1680 | 54.5 | 534 | 39.4 |
| <i>S. cerevisiae</i> "Gebruder Mayer" | | | | | | |
| 4 | 0.39 | 2.30 | 19 | 38.5 | 87 | 34.7 |
| 8 | 1.17 | 6.70 | 100 | 21.3 | 107 | 28.8 |
| 12 | 1.84 | 10.10 | 240 | 19.0 | 150 | 25.0 |
| 16 | 2.48 | 10.15 | 460 | 17.7 | 203 | 26.6 |
| 24 | 2.98 | 10.15 | 900 | 16.1 | 318 | 20.6 |
| <i>M. lipolytica</i> P-13 | | | | | | |
| 4 | 0.16 | 0.35 | 9 | 48.8 | 102 | 31.3 |
| 8 | 0.35 | 1.30 | 32 | 27.1 | 118 | 24.3 |
| 12 | 1.32 | 4.00 | 189 | 17.4 | 160 | 51.2 |
| 16 | 3.04 | 5.46 | 660 | 8.5 | 226 | 54.5 |
| 24 | 5.82 | 11.10 | 1680 | 7.7 | 295 | 29.3 |

TABLE I—*Continued*

| Fermentation | Dry cells | Glucose used | PAB content | | Total PAB synthesis | Average PAB synthesis/ g./hr. at each interval |
|-----------------------|-----------|--------------|-------------------------------|----------|---------------------|---|
| | | | Of fermented cell-free medium | Of cells | | |
| <i>H. anomala</i> 778 | | | | | | |
| hr. | g./l. | g./l. | μg./l. | μg./g. | μg./g. | μg. |
| 4 | 0.31 | 1.80 | 20 | 12.3 | 77 | 26.5 |
| 8 | 1.18 | 4.30 | 43 | 7.3 | 44 | 9.4 |
| 12 | 2.52 | 6.90 | 62 | 5.9 | 30 | 3.4 |
| 16 | 3.96 | 9.77 | 80 | 4.0 | 24 | 1.5 |
| 24 | 4.74 | 10.15 | 92 | 1.8 | 21 | 0.1 |
| <i>C. krusoides</i> | | | | | | |
| 4 | 0.32 | 1.80 | 10 | 20.6 | 50 | 18.0 |
| 8 | 1.20 | 3.80 | 26 | 9.1 | 33 | 7.2 |
| 12 | 2.70 | 5.24 | 60 | 1.9 | 24 | 3.5 |
| 16 | 4.30 | 8.95 | 100 | 1.5 | 25 | 2.9 |
| 24 | 3.94 | 10.15 | 105 | 1.2 | 28 | 1.2 |

^a The inoculum added (g./l.) was: *S. cerevisiae* Y-30 0.10, *S. cerevisiae* G. M. 0.10, *M. lipolytica* 0.10, *H. anomala* 0.14, and *C. krusoides* 0.12. In calculation of the PAB synthesis between 0-4 hr. the PAB in the inoculum has been neglected.

also show that as the weight of cells increased, there was a greater PAB content of the medium.

The young cells of *S. cerevisiae* Y-30 were much lower in PAB than older cells. At 4 hr., they contained 6.0 μg./g., while at 24 hr. a value of 54.5 μg./g. was reached. An exactly reverse situation was observed with the other four yeasts. The cells continuously decreased in PAB content as they grew older. There is no evident reason why *S. cerevisiae* Y-30 should differ from the other strains of *S. cerevisiae*, but a high PAB content in this yeast has been noted many times. For example, in synthetic medium the figures are 39 μg. in Table III and 28.4 μg. in Table V.

Column 6 of Table I gives the amount of PAB synthesized per gram at different periods of growth. With the three high producers of PAB there is a gradual increase in the values at the successive periods. An increase of about 6-fold was observed for *S. cerevisiae* Y-30, about 3.5-

fold for *S. cerevisiae* "Gebruder Mayer," and about 3-fold for *M. lipolytica*. On the other hand, *H. anomala* and *C. krusoides* (two low producers of PAB) showed a decrease from 77 to 21 $\mu\text{g.}$ and 50 to 28 $\mu\text{g.}$, respectively. In order to compare the rate of production of PAB at each interval, average values for the synthesis per gram of cells per hour were calculated, and are presented in the last column of the table. A sample calculation for *S. cerevisiae* Y-30 between 4-8 hr. is as follows:

$$\begin{aligned}\text{Average amount of cells during that period} &= (0.28 + 0.75)/2 \\ &= 0.515 \text{ g./l.}\end{aligned}$$

$$\begin{aligned}\text{PAB synthesis in 1 hr.} &= [(.75 \times 7 + 76) - (.28 \times 6 + 24)]/4 \\ &= 13.9 \mu\text{g./l.}\end{aligned}$$

$$\begin{aligned}\text{Hence average synthesis/g./hr. at this interval} \\ &= 13.9/0.515 = 26.7 \mu\text{g.}\end{aligned}$$

In calculating the values between 0-4 hr., the PAB in the inoculum cells has been neglected. The values for *S. cerevisiae* Y-30 and *M. lipolytica* P-13 were lowest between 4-8 hr. and highest between 12-16 hr. A considerable decrease was noted between 16-24 hr. with these two yeasts, which may be related to decreased supply of nutrients and consequent cell development. The values for *S. cerevisiae* G. M., *H. anomala*, and *C. krusoides* continuously decreased as the period of fermentation increased; *i.e.*, there was greater synthesis of PAB during the early stages of fermentation.

PAB Synthesis Under Commercial Conditions

Samples of two kinds of bakers' yeast (active dry and press yeast) were obtained from the Red Star Yeast and Products Co., Milwaukee. The samples were taken at different times during the fermentation period, were kept cold during shipment, and reached the laboratory only a few hours after being collected. The data on these samples are given in Table II.

Since wort is fed in during the fermentation, the volume in the fermenter is constantly changing and therefore the data are expressed in terms of weight of yeast. In the production of active dry yeast, the PAB content of the cells decreased from 36 $\mu\text{g./g.}$ at the start of fermentation to 28 $\mu\text{g.}$ at the end of the first quarter, then rose to 58 $\mu\text{g.}$ at the halfway mark, reached a maximum, 69 $\mu\text{g.}$ at three-quarters of the period, and then dropped to 62 $\mu\text{g.}$ at the end. The PAB content of

the cell-free medium rose steadily throughout the fermentation. In round numbers, only about 20% of the PAB remained in the cell while 80% passed into the medium.

In the production of bakers' press yeast, about 30% more PAB was synthesized than in the making of active dry yeast. More than 90% of this was released into the medium. In another run of bakers' yeast,

TABLE II
Synthesis of p-Aminobenzoic Acid by Yeast Under Commercial Conditions

| Time of fermentation | PAB, dry yeast basis | | | Distribution in | |
|----------------------------|----------------------|-------------------|-------------------|-----------------|-------|
| | In cell-free medium | In yeast | Total | Medium | Yeast |
| <i>Active dry yeast</i> | | | | | |
| h. | $\mu\text{g./g.}$ | $\mu\text{g./g.}$ | $\mu\text{g./g.}$ | $\%$ | $\%$ |
| 0 | 11 | 36 | 47 | 23 | 77 |
| 5.25 | 180 | 28 | 208 | 87 | 13 |
| 10.5 | 204 | 58 | 262 | 78 | 22 |
| 15.8 | 273 | 69 | 342 | 80 | 20 |
| 21 | 277 | 62 | 339 | 82 | 18 |
| <i>Bakers' press yeast</i> | | | | | |
| 0 | 38 | 57 | 95 | 40 | 60 |
| 5 | 260 | 18 | 278 | 94 | 6 |
| 10 | 312 | 39 | 351 | 89 | 11 |
| 15 | 438 | 27 | 465 | 94 | 6 |
| 20 | 423 | 21 | 444 | 95 | 5 |

the cells contained 46 $\mu\text{g.}$ PAB/g. dry matter, and the cell-free medium corresponding to 1 g. of dry yeast contained 453 $\mu\text{g.}$ The distribution here was 9% in the yeast and 91% in the medium. The data on the two lots of commercial bakers' yeast are in the same range as was found for the laboratory run of *S. cerevisiae* Y-30 (Table I).

Effect of Concentration of Sugar on PAB Synthesis

The effect of concentration of sugar on the growth and PAB production of *Saccharomyces cerevisiae* and *Mycotorula lipolytica* is given

in Table III. As has been frequently observed, higher yields of yeast, based on sugar fermented, were obtained at the lower concentration of sugar. *S. cerevisiae* used almost all the sugar even at levels of 4%, but only a 64.1% utilization was observed at 10% concentration. The weight of cells increased with the increment of sugar, until a maximum of 4.62 g./l. was reached at 4% sugar, and then at higher levels a lower

TABLE III
Effect of Initial Concentration of Sugar on the PAB Synthesis by Some Yeasts in Synthetic Medium^a

| Glucose in medium | Glucose used | Dry cells | Yield of Yeast | PAB content | | Total PAB synthesis | |
|---------------------------|--------------|-----------|----------------|-------------------------------|----------|---------------------|--------------|
| | | | | Of fermented cell-free medium | Of cells | | |
| <i>S. cerevisiae</i> Y-30 | | | | | | | |
| % | % | g./l. | % | μg. l. | μg. g. | μg. l. | μg./g. cells |
| 1 | 99.3 | 3.58 | 30.5 | 1260 | 39.0 | 1400 | 391 |
| 2 | 99.1 | 4.16 | 18.4 | 1520 | 23.5 | 1620 | 390 |
| 4 | 98.5 | 4.62 | 10.0 | 1650 | 5.4 | 1680 | 364 |
| 7 | 71.2 | 4.40 | 7.1 | 1460 | 1.8 | 1470 | 335 |
| 10 | 64.1 | 4.24 | 6.0 | 1200 | 1.4 | 1210 | 202 |
| <i>M. lipolytica</i> P-13 | | | | | | | |
| 1 | 99.4 | 4.74 | 43.0 | 2300 | 11.8 | 2360 | 498 |
| 2 | 94.1 | 6.96 | 34.5 | 4030 | 30.9 | 4240 | 610 |
| 4 | 51.8 | 6.90 | 29.9 | 4830 | 45.6 | 5140 | 750 |
| 7 | 34.3 | 6.80 | 26.9 | 5000 | 47.0 | 5320 | 785 |
| 10 | 22.3 | 5.98 | 25.6 | 4750 | 12.4 | 4820 | 810 |

^a Fermentations were run for 24 hr. Yield of yeast (Col. 4) is based on weight of dry yeast obtained from sugar fermented.

yield was obtained. With *M. lipolytica*, the greatest yield of cells was obtained at the 2% level and then there was a continuous decrease till the lowest of 5.98 g. was reached at the 10% level. Synthesis of PAB by both yeasts increased simultaneously with an increase in weight of the cells and reached a maximum at ca. 2-4% sugar fermentation. However, maximum sugar utilization by *S. cerevisiae* was reached at the

10% level. At this high level the anaerobic type of metabolism, *i.e.*, alcohol production, probably becomes predominant and synthetic processes, *i.e.*, cell production and PAB synthesis, are depressed.

It is also evident from Table III that concentration of sugar has a significant effect on the amount of PAB retained in the cells. With *S. cerevisiae*, there was a marked decrease in the amount of PAB in the cells as the concentration of sugar in the medium was increased. The cells grown in 1% sugar contained 39.0 $\mu\text{g./g.}$ compared with 1.4 $\mu\text{g./g.}$, when the yeast was cultured in 10% glucose. The data for *M. lipolytica* show a reverse situation. At 1% concentration only 11.8 $\mu\text{g./g.}$ was retained in the cells, while at 7%, 47 $\mu\text{g.}$ was found. A possible explanation for the variation in PAB content of *S. cerevisiae* may lie in the different types of metabolism that take place at high and low concentrations of sugar. At the high levels alcoholic fermentation rather than cell-building predominates. At the low level the reverse is true. It does not appear probable that *M. lipolytica* inaugurates alcohol production at the higher concentration of sugar as sugar utilization is not increased appreciably.

Effect of Kind of Sugar on PAB Synthesis

Since pentoses can be used by some yeasts, *e.g.*, *Torula utilis* and *Candida kruseoides*, for cell growth, and since xylose is contained in wood sugar and sulfite waste liquor, which are or may be used for the industrial production of yeast (10,11,12), the effect of its substitution for glucose in the synthetic medium on the yield of cells and PAB synthesis was studied. Four yeasts, *Candida kruseoides*, *Hansenula anomala* 778, *Rhodotorula gracilis* and *Torula utilis* 3, were selected for this study because they have the ability to use xylose. The data are presented in Table IV. It is evident that the yield of cells is much lower in xylose than in glucose medium. Xylose utilization was complete in fermentations with *C. kruseoides* and *T. utilis*, but was only 25% and 30% with *H. anomala* and *R. gracilis*, respectively. Even though the amount of cells produced was less in xylose, more PAB was produced, as indicated in the last column of the table. *C. kruseoides* synthesized 280 $\mu\text{g./g.}$ in xylose medium compared with 29 $\mu\text{g./g.}$ in glucose medium. The other three yeasts also showed a sixfold to tenfold increase in the PAB synthesis, when glucose was replaced by xylose. It is also interesting that the cells produced from xylose are higher in their PAB content than those produced from glucose. *H. anomala* and *R. gracilis* showed the

greatest increase in this respect. The former increased from 4.8 to 22.7 $\mu\text{g./g.}$, and the latter from 3.0 to 17.8 $\mu\text{g./g.}$

A possible explanation for the greater synthesis in xylose may be that the yeasts synthesize and retain more PAB when grown under unfavorable circumstances; for the complete establishment of this theory other unfavorable factors for yeast growth must be tried.

Effect of Added PAB on the PAB Synthesis

Since PAB production varies under different conditions, it was thought possible that addition of excess PAB might stop the production of this

TABLE IV
Effect of Sugar on the PAB Synthesis by Some Yeasts^a

| Yeast | Sugar | Yield on fermented sugar | Dry cells | PAB content | | Total PAB synthesis |
|---------------------|---------|--------------------------|---------------|-------------------------------|---------------------------|---------------------------------|
| | | | | Of fermented cell-free medium | Of cells | |
| <i>C. krusoides</i> | Xylose | % 28.2 | g./l. 2.84 | $\mu\text{g./l.}$ 760 | $\mu\text{g./g.}$ 14.7 | $\mu\text{g., g. cells}$ 280 |
| <i>C. krusoides</i> | Glucose | 36.9 | 3.76 | 90 | 4.8 | 29 |
| <i>H. anomala</i> | Xylose | 20.5 | 1.10 | 180 | 22.7 | 180 |
| <i>H. anomala</i> | Glucose | 46.1 | 4.76 | 80 | 4.8 | 21 |
| <i>R. gracilis</i> | Xylose | 21.9 | 0.84 | 58 | 17.8 | 85 |
| <i>R. gracilis</i> | Glucose | 34.7 | 4.16 | 50 | 3.0 | 15 |
| <i>T. utilis</i> 3 | Xylose | 35.3 | 3.96 | 360 | 13.9 | 104 |
| <i>T. utilis</i> 3 | Glucose | 50.8 | 4.06 | 20 | 4.7 | 10 |

^a Fermentations were run for 24 hr., 1% sugar was used, and all the vitamins were added in both cases.

vitamin. Fermentations with eight different yeasts were set up with an initial PAB addition of 2000 $\mu\text{g./l.}$ in the medium. The results are presented in Table V. The yield of all the yeasts tested was about the same with or without added PAB, as indicated by the dry weight of cells given in Col. 3. From the last column it is evident that there was marked reduction of PAB synthesis when a large quantity of PAB was added to the medium. This conclusion is valid for *S. cerevisiae* "Gebrüder Mayer," *S. cerevisiae* "Old Process," and *M. lipolytica* P-13, the last-named yeast showing a reduction of about 69%. With the other

TABLE V

Effect of Added PAB on the Synthesis of PAB by Some Yeasts in the Synthetic Medium^a

| Yeast | PAB added | Dry cells | PAB content | | Total PAB synthesis | Per cent of original synthesis |
|----------------------------|-------------------|----------------|-------------------------------|-------------------|---------------------|--------------------------------|
| | | | Of fermented cell-free medium | Of cells | | |
| | $\mu\text{g./l.}$ | g./l. | $\mu\text{g./l.}$ | $\mu\text{g./g.}$ | $\mu\text{g./l.}$ | |
| <i>S. cerevisiae</i> 53 | 0 | 2.16 | 320 | 2.7 | 330 | — |
| <i>S. cerevisiae</i> 53 | 2000 | 2.24 | 2260 | 4.9 | 270 | 82 |
| <i>S. cerevisiae</i> Y-30 | 0 | 3.10 | 1660 | 28.4 | 1750 | — |
| <i>S. cerevisiae</i> Y-30 | 2000 | 3.10 | 3360 | 45.2 | 1500 | 81 |
| <i>S. cerevisiae</i> G. M. | 0 | 3.44 | 1600 | 24.6 | 1680 | — |
| <i>S. cerevisiae</i> G. M. | 2000 | 3.40 | 2840 | 46.1 | 1100 | 66 |
| <i>S. cerevisiae</i> O. P. | 0 | 2.20 | 1800 | 7.3 | 1820 | — |
| <i>S. cerevisiae</i> O. P. | 2000 | 2.44 | 3000 | 13.5 | 1030 | 56 |
| <i>M. lipolytica</i> P-13 | 0 | 5.20 | 1800 | 20.0 | 1900 | — |
| <i>M. lipolytica</i> P-13 | 2000 | 4.86 | 2460 | 24.6 | 580 | 31 |
| <i>C. krusoides</i> | 0 | 4.08 | 100 | 10.1 | 140 | — |
| <i>C. krusoides</i> | 2000 | 4.08 | 1800 | 53.9 | 20 | 14 |
| <i>T. utilis</i> 1 | 0 | 3.72 | 140 | 4.0 | 155 | — |
| <i>T. utilis</i> 1 | 2000 | 3.78 | 1940 | 15.0 | 0 | 0 |
| <i>T. utilis</i> 3 | 0 | 5.80 | 2.2 | 1.4 | 10 | — |
| <i>T. utilis</i> 3 | 2000 | 5.86 | 1920 | 14.0 | 0 | 0 |

^a Yeast grown for 24 hrs.

organisms tested, an apparent reduction in PAB synthesis was observed, but the results were not beyond the limits of experimental error.

It may also be noted that cells grown in media to which PAB was added were higher in PAB content than those grown without the addition. Cells of *C. krusoides* showed a fivefold increase, from 10.1 to 53.9 $\mu\text{g./g.}$ Maximum difference was observed with *T. utilis* 3, in which the PAB content increased from 1.4 to 14 $\mu\text{g./g.}$, i.e., the cells grown with added PAB were ten times as rich. A possible explanation for this observation may be that the high concentration of PAB in the medium may prevent its diffusion out of the cells.

Effect of Vitamin on PAB Synthesis

Since vitamins play such an important role in the metabolism of cells it is not unreasonable to assume that they may be involved in the

synthesis of PAB. Therefore, experiments with and without vitamins (thiamine 4 mg./l., pyridoxine 1 mg., biotin 20 μ g., pantothenate 0.5 mg., niacin 4 mg., and inositol 10 mg.) were set up, and the results are presented in Table VI. The strains of *S. cerevisiae* have been omitted from the table, because they require some of these vitamins for growth, and a comparison obviously could not be made. It is evident that the yields of yeast were not much affected by omission of the vitamins. An exception was observed with *C. krusoides* with which the addition of vitamins gave a 30% higher yield. All yeasts showed a greater PAB

TABLE VI
Effect of Vitamins on the PAB Synthesis by Some Yeasts^a

| Yeast | Yield on fermented sugar | Dry cells | PAB content | | Total PAB synthesis | |
|-------------------------|--------------------------|-----------|-------------------------------|-------------|---------------------|-------------------|
| | | | Of fermented cell-free medium | Of cells | | |
| | % | g./l. | μ g./l. | μ g./g. | μ g./l. | μ g./g. cells |
| <i>C. krusoides</i> | 30.1 | 3.66 | 170 | 4.9 | 188 | 51.4 |
| (<i>C. krusoides</i>) | 23.8 | 2.82 | 700 | 13.5 | 738 | 262 |
| <i>R. gracilis</i> | 27.5 | 2.54 | 3.0 | 0.63 | 4.6 | 1.8 |
| (<i>R. gracilis</i>) | 25.9 | 2.58 | 15 | 0.47 | 16.2 | 6.3 |
| <i>T. utilis</i> 2 | 40.7 | 4.84 | 4.1 | 2.2 | 14.6 | 3.0 |
| (<i>T. utilis</i> 2) | 40.5 | 4.82 | 60 | 2.9 | 74.0 | 15.3 |
| <i>T. utilis</i> 3 | 40.1 | 4.82 | 2.6 | 1.1 | 8.1 | 1.7 |
| (<i>T. utilis</i> 3) | 39.8 | 4.66 | 10 | 1.1 | 15.4 | 3.3 |

^a The yeasts were run in the synthetic medium for 24 hr. The yeasts in parentheses were grown without vitamins.

production when the vitamins were left out of the medium, the increase being different with individual yeasts. *C. krusoides* and *T. utilis* 2 showed a fivefold increase in the synthesis of PAB per gram (Col. 7), while *T. utilis* 3 and *R. gracilis* produced, respectively, only about two and three times that formed in the presence of vitamins. The cells of *C. krusoides* were also richer in PAB content, assaying 13.5 μ g./g. without vitamins as compared with 4.9 μ g./g. when grown with vitamins. The cells of the other yeasts did not show any appreciable difference.

Two yeasts, *C. krusoides* and *T. utilis* 2, which showed maximum difference in the preceding experiment were selected for further study. Fermentations were run with and without individual vitamins and the

results are given in Table VII. For both organisms, addition of thiamine had the greatest influence on PAB production. With *C. krusoides*, addition of 4.0 mg./l. of thiamine reduced the PAB synthesis to about one-third of the original amount. With *T. utilis* 2, the amount of PAB synthesized was reduced from 62 $\mu\text{g.}/\text{l.}$ to 14 $\mu\text{g.}/\text{l.}$ A possible explanation for the reduction of PAB synthesis is that PAB is involved in the synthesis of thiamine, and addition of thiamine reduces the need for PAB.

TABLE VII

Effect of Addition of Individual Vitamins on the PAB Synthesis by Yeasts^a

| Medium | Yield on fermented sugar | Dry cells | PAB content | | Total PAB synthesis | |
|---------------------|--------------------------|-----------|-------------------------------|--------------------------|--------------------------|--------------------------------|
| | | | Of fermented cell-free medium | Of cells | | |
| | % | g./l. | $\mu\text{g.}/\text{l.}$ | $\mu\text{g.}/\text{g.}$ | $\mu\text{g.}/\text{l.}$ | $\mu\text{g.}/\text{g. cells}$ |
| <i>C. krusoides</i> | | | | | | |
| no. vitamins | 30.0 | 2.82 | 340 | 3.7 | 350 | 124 |
| + thiamine | 32.3 | 3.02 | 115 | 3.0 | 124 | 41 |
| + pyridoxine | 34.4 | 3.20 | 310 | 8.1 | 336 | 105 |
| + biotin | 27.7 | 2.62 | 350 | 1.9 | 355 | 135 |
| + pantothenic acid | 34.6 | 3.22 | 340 | 11.8 | 378 | 117 |
| + niacin | 29.9 | 2.80 | 330 | 5.0 | 344 | 123 |
| + inositol | 29.6 | 2.76 | 360 | 5.8 | 376 | 137 |
| <i>T. utilis</i> 2 | | | | | | |
| no vitamins | 55.9 | 5.24 | 43 | 3.6 | 62 | 11.8 |
| + thiamine | 53.4 | 5.02 | 3 | 2.2 | 14 | 2.7 |
| + pyridoxine | 56.3 | 5.28 | 17 | 3.3 | 34 | 6.4 |
| + biotin | 55.4 | 5.20 | 56 | 3.2 | 73 | 14.0 |
| + pantothenic acid | 55.4 | 5.20 | 43 | 3.0 | 59 | 11.3 |
| + niacin | 52.1 | 4.90 | 38 | 2.9 | 52 | 10.6 |
| + inositol | 52.7 | 4.96 | 64 | 6.2 | 95 | 19.2 |

^a The fermentations were run for 24 hr. in the synthetic medium.

On the other hand, the thiamine-rich cells may have a different metabolism and synthesize less PAB. The possibility of the cells utilizing PAB more efficiently in the presence of thiamine, and thus leading to lower production, likewise cannot be overlooked.

The other vitamin which appears to play a role in PAB production is pyridoxine. No appreciable reduction in the synthesis of PAB by addition of pyridoxine was noted with *C. krusoides*, but with *T. utilis* 2

the PAB formed in the presence of added pyridoxine was only about one-half that produced in its absence. Cells of *C. krusoides* grown in the presence of pyridoxine were richer in PAB than those grown without it. With *T. utilis* 2, no difference was observed.

In contrast to thiamine and pyridoxine, inositol appeared to increase production of PAB by *T. utilis*. The other vitamins did not appear to play any part in PAB synthesis as their addition or removal did not make any significant difference.

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SUMMARY

Synthesis of *p*-aminobenzoic acid (PAB) by yeasts varied with species, strain, and nutrients. *S. cerevisiae* and *M. lipolytica* are high producers and *T. utilis*, *R. gracilis*, *H. anomala*, and *C. krusoides* give low yields. Strains of the same species, e.g., *S. cerevisiae*, varied greatly in ability to synthesize PAB.

Increasing the concentration of glucose had no particular effect on PAB synthesis by *S. cerevisiae* Y-30 but doubled the production by *M. lipolytica* P-13.

C. krusoides, *H. anomala*, *R. gracilis*, and *T. utilis* utilize xylose, but a lower yield of cells was obtained on it than on glucose. A higher PAB synthesis was observed on the xylose medium. Cells grown on xylose were also richer in PAB than those produced from glucose.

Addition of excess PAB to the medium reduced its synthesis from 18 to 100%, but the amount retained in the cells was markedly increased.

A depression of PAB synthesis with four yeasts was observed by the addition of vitamins to the medium. The most significant difference resulted from the addition of thiamine. Reductions in PAB synthesis from 124 $\mu\text{g./g.}$ to 41 $\mu\text{g./g.}$ and from 11.8 to 2.7 $\mu\text{g.}$ were observed with *C. krusoides* and *T. utilis*, respectively. These data suggest that PAB plays a role in the synthesis of thiamine. Another vitamin which brought a smaller decrease in the production of PAB was pyridoxine.

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Effect of Electrolytes on Cholinesterase Inhibition

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INTRODUCTION

As shown by Mendel and Rudney, the affinity of the true cholinesterase for acetylcholine is decreased very considerably by the presence of electrolytes (1). On this basis it might be expected that similarly the degree of inhibition brought about by competitive inhibitors of the cholinesterases might change with alterations in the electrolytic environment. The experiments outlined below were designed to investigate this problem, and it was found that the concentration of inhibitor required to produce 50% inhibition of cholinesterase activity (I_{50}) was in fact often increased two to five times by the addition of salts to a bicarbonate medium.

METHODS

In all cases the media used contained 0.025 *M* sodium bicarbonate and were saturated with 5% CO_2 to give a pH of 7.4 at 37°C., this pH being in the physiological range and near the optimum pH of the esterases tested (2). To determine the effect of a more physiological salt solution, a medium was used containing 0.15 *M* NaCl, 0.003 *M* KCl, and 0.002 *M* CaCl_2 , in addition to the bicarbonate. Dialyzed hemolyzed human erythrocytes were used as the source of true cholinesterase and dialyzed human plasma as the source of pseudocholinesterase; the activities of these two enzymes were determined by a manometric technique using acetyl- β -methylcholine (0.03 *M*) and benzoylcholine (0.006 *M*) as respective substrates (3). The degree of inhibition produced by incubation of the enzyme with a series of concentrations of inhibitor was estimated from the activity measured in the period 2–12 min. after addition of substrate, results so obtained will give an estimate of the degree of noncompetitive inhibition of the enzyme, but, as pointed out by Goldstein (4), there will be some distortion of the results, due mainly to displacement of the inhibitor by substrate during the period of measurement.

The value of pI_{50} ($-\log I_{50}$), as determined under the conditions outlined above, can be taken as a convenient experimental index of the enzyme-inhibitor dissociation constant K_I providing that the value of pI_{50} is independent of enzyme concentration

(zone A) (6). Values of the slope, $d(a)/d(pI)$, obtained in the neighborhood of pI_{50} from plots relating fractional activity "a" and pI , were also determined for various inhibitors. As shown by Goldstein (4), this value should be 0.575 if the enzyme-inhibitor system is in zone A and the mechanism of inhibition is such that one molecule of inhibitor combines reversibly with each active center of the enzyme. In most cases tested, the experimental evidence obtained (Table II) would justify this latter assumption.

EXPERIMENTAL RESULTS

In agreement with the predictions of Goldstein (4), the complete equilibration of eserine with pseudocholinesterase preparations was found to be a moderately slow process, even in the absence of substrate. This can be seen from the experiments outlined in Table I, where substrate was added after various periods of incubation to stop further noncompetitive combination of enzyme with inhibitor. To make certain that an apparent decrease in affinity of inhibitor for pseudocholinesterase in the presence of a high salt concentration was not due merely to a slower rate of combination of enzyme and inhibitor in the presence of salt, the cholinesterase of plasma was equilibrated with eserine in Ringer's bicarbonate for 25–150 min.; it was found that the ratio of I_{50} values (I_{50} in Ringer's bicarbonate/ I_{50} in 0.025 *M* bicarbonate) was still 2.6 after 150 min., as compared with a value of 2.8 after 25 min. incubation. In all subsequent experiments the inhibitor was incubated with the enzyme for approximately 30 min. at 37°C. before adding substrate, a period long enough to ensure relatively complete equilibration of enzyme with inhibitor but not long enough to allow any appreciable destruction of inhibitor to take place. The increase in the values of slope and I_{50} following 150 min. incubation of enzyme with eserine

TABLE I
*Time Course of Noncompetitive Combination of Eserine
with True and Pseudocholinesterase*

| Time of incubation of enzyme with eserine at 37° | Pseudocholinesterase | | True cholinesterase | |
|--|----------------------|----------------------------|---------------------|----------------------------|
| | pI_{50} | Slope $\frac{d(a)}{d(pI)}$ | pI_{50} | Slope $\frac{d(a)}{d(pI)}$ |
| <i>min.</i> | | | | |
| 10 | 7.95 | 0.59 | 8.27 | 0.60 |
| 25 | 8.25 | 0.625 | 8.53 | 0.583 |
| 65 | 8.28 | 0.646 | 8.58 | 0.563 |
| 150 | 8.18 | 0.723 | 8.42 | 0.645 |

TABLE II

Effect of Altering the Salt Concentration of the Medium on the Sensitivity of the Cholinesterases to Various Inhibitors

| Enzyme preparation | Inhibitor | pI_{50} | | Slope $\frac{d(a)}{d(pI)}$ | Ratio of I_{50} values |
|--------------------|-------------|---------------------|----------------------|----------------------------|--------------------------|
| | | 0.025 M bicarbonate | Ringer's bicarbonate | | |
| Erythrocytes (1½%) | Eserine | 8.5 | 7.9 | 0.57 | 3.9 |
| Plasma (3%) | Eserine | 8.2 | 7.7 | 0.62 | 2.8 |
| Erythrocytes | Prostigmine | 8.4 | 7.8 | 0.63 | 4.0 |
| Plasma | Prostigmine | 7.3 | 6.8 | 0.60 | 3.1 |
| Erythrocytes | Nu-1250 | 8.8 | 8.4 | 0.63 | 2.9 |
| Plasma | Nu-1250 | 4.1 | 4.1 | 0.21 ^a | 1.0 ^a |
| Erythrocytes | Nu-683 | 7.6 | 7.0 | 0.56 | 4.2 |
| Plasma (½%) | Nu-683 | 9.0 | 8.5 | 0.67 | 3.3 |
| Plasma (3%) | Nu-683 | 8.7 | 8.4 | 0.85 | 1.8 |
| Plasma (20%) | Nu-683 | 8.2 | 8.1 | 1.06 | 1.2 |
| Erythrocytes | DFP | 7.1 | 7.1 | 0.90 | 1.0 |
| Plasma (½%) | DFP | 8.9 | 9.0 | 0.70 | 1 |
| Plasma (3%) | DFP | 8.7 | 8.8 | 0.86 | 1 |
| Plasma (20%) | DFP | 8.0 | 8.0 | 1.00 | 1 |

^a As judged by the value of the slope $\frac{d(a)}{d(pI)}$ at $pI = pI_{50}$, the inhibition of pseudo-cholinesterase by Nu-1250 would not appear to conform to the same mechanism of combination as outlined for eserine (4) but was found, rather, to be partially non-competitive in nature, one molecule of inhibitor combining with at least two enzyme molecules.

(Table I) demonstrates that this factor of destruction of inhibitor may modify the results significantly when long periods of incubation are employed.

The effect of altering the electrolyte concentration of the medium on the sensitivity of cholinesterases to various inhibitors is indicated in Table II. It will be seen that alterations in the salt concentration of the medium cause a shift in pI_{50} values in the case of eserine, prostigmine, and prostigmine analogs. Representative experimental data are also given in Table III, showing that a higher concentration of inhibitor is

required in the presence of sodium chloride than in its absence to bring about the same inhibition of cholinesterase activity.

To justify the assumption that a change in pI_{50} on addition of electrolytes as determined above is evidence of a real change in the value of K_1 , the possible effect of various other factors on the values of pI_{50} had to be excluded. Using erythrocyte true cholinesterase as enzyme source and eserine as inhibitor, it was found that substitution of acetylcholine (activity measured during the period 1.5–6 min. following addition of substrate) for acetyl- β -methylcholine as substrate, or a change

TABLE III
Effect of Sodium Chloride on the Inhibition of True Cholinesterase Activity^a Brought About by Various Concentrations of Eserine

| Concentration of eserine | Medium: 0.025 M sodium bicarbonate | | Medium 0.025 M sod. bicarb. plus 0.125 M sod. chloride | |
|--------------------------|------------------------------------|---------------------------|--|---------------------------|
| | Activity | Inhibition due to eserine | Activity | Inhibition due to eserine |
| $\times 10^{-9}$ M | μ l. | per cent | μ l | per cent |
| 0 | 86.1 | — | 85.4 | — |
| 1 | 68.0 | 21 | — | — |
| 2 | 54.1 | 37 | — | — |
| 3 | 48.0 | 44 | 72.0 | 16 |
| 5 | 33.3 | 61 | 61.0 | 28 |
| 9 | 24.0 | 72 | 47.9 | 44 |
| 14 | — | — | 36.6 | 57 |
| 20 | — | — | 30.0 | 65 |

^a Activity expressed as μ l. CO_2 produced in the period 2–17 min. after addition of acetyl- β -methylcholine to the enzyme-inhibitor mixture (previously incubated for 30 min. at 37°), using 0.2 ml. of 3-fold diluted human erythrocytes as the source of true cholinesterase.

in substrate concentration from 6×10^{-4} M to 4×10^{-3} M acetylcholine, or reduction of the enzyme concentration to $\frac{1}{4}$ of that previously used did not cause any appreciable change in the value of pI_{50} . Thus the use of these values of pI_{50} as a comparative measure of K_1 seems justified. Further, a fiftyfold purification of the enzyme (5) had no measurable effect on the activity- pI curves obtained in the absence or presence of a large electrolyte concentration, thereby rendering any appreciable nonspecific effect of other proteins less likely. Varying the

pH by changing the bicarbonate concentration but maintaining a constant total sodium-ion concentration of 0.15 *M* in the medium caused only a very slight shift in pI_{50} (8.05 at pH 6.6, 7.97 at pH 7.4, and 7.82 at pH 7.9), thus ruling out alterations in the degree of ionization of the bicarbonate as the responsible factor. No change in the activity- pI curve was observed when intact erythrocytes were substituted for hemolyzed erythrocytes, thereby excluding permeability factors from being involved in the observed phenomenon. All these results would support the conclusion that the effect of salts on the dissociation constant of the enzyme-inhibitor system is, therefore, not merely some indirect effect on other characteristics of the reaction mixture.

The results outlined in Table IV demonstrate that the addition of increasing concentrations of each of the individual salts in the Ringer's solution induces changes in the affinity of the enzyme for the inhibitor

TABLE IV

Effect of Individual Salts on Value of pI_{50} with True Cholinesterase and Eserine

| Added concentration of NaCl | pI_{50} | Added concentration of KCl | pI_{50} | Added concentration of $CaCl_2$ | pI_{50} |
|-----------------------------|-----------|----------------------------|-----------|---------------------------------|-----------|
| 0.000 | 8.50 | — | — | — | — |
| 0.045 | 8.17 | — | — | 0.001 | 8.24 |
| 0.125 | 7.94 | 0.125 | 7.94 | 0.004 | 8.01 |
| 0.475 | 7.72 | — | — | 0.015 | 7.78 |

analogous to the changes seen in the affinity of the true cholinesterase for acetylcholine (1), with a disproportionately large effect by such divalent cations as calcium ions. That this is the case in spite of the disparity in the concentration of inhibitor on the one hand and substrate on the other also substantiates the assumption that the change in pI_{50} caused by the addition of salts represents a direct effect of these salts on the dissociation constant of the enzyme-inhibitor system.

Similarly, the experimental results with Nu-683 and plasma pseudo-cholinesterase (Table II) are in accordance with the general mechanisms of enzyme-inhibitor combination outlined by Strauss and Goldstein (6) for reversible competitive inhibitors, if the assumption is made that the effect of added electrolytes is only to decrease the affinity of the inhibitor for the cholinesterase. Thus, in dilute plasma solutions where the enzyme-inhibitor system is in zone A and the value of I_{50} depends

mainly on the dissociation constant K_I , the addition of salts increases the value of I_{50} for the pseudocholinesterase Nu-683 system three- to fourfold, whereas in more concentrated plasma solutions in which the system is in zone *C* and the value of I_{50} depends mainly on enzyme concentration, the addition of salts had almost no effect on the value of I_{50} .

Similar tests with diisopropyl fluorophosphate (DFP), an irreversible but competitive inhibitor of cholinesterases (7,8), did not show an analogous effect of salts upon the value of pI_{50} , however. The initial phase of this inhibition is known to involve a reversible association of enzyme and inhibitor (7) and the curves obtained with DFP do simulate to some extent those expected with reversible competitive inhibitors (8), but it has been found in this investigation that the rate of inactivation of cholinesterase by DFP in the absence of substrate is not appreciably altered at any time by the presence of salts, even when the enzyme has been incubated with the salt-containing medium for 20–30 min. prior to addition of the inhibitor.

DISCUSSION

Using the method outlined above, it can be demonstrated that the affinity of the cholinesterases for such reversible competitive inhibitors as eserine and prostigmine is decreased when the concentration of salts in the medium is increased. If we assume that the combination of true cholinesterase with acetylcholine involves an association between substrate and at least two principal points of the enzyme active center, as proposed by Zeller *et al.* (9), and that one of these points is probably associated with the ester linkage of the substrate while the other, presumably a negatively-charged position on the enzyme surface, becomes associated with the quaternary nitrogen of choline esters, we might then postulate that the effect of electrolytes is to compete with the cationic portion of substrate or inhibitor for the anionic position of the enzyme active center. Thus the effect of salts would be to decrease the affinity of the true cholinesterase for acetylcholine, eserine, prostigmine, and analogous compounds. The fact that electrolytes have no effect upon the affinity of DFP for cholinesterases might then be interpreted as meaning that DFP combines only with the "ester-grouping" of the enzyme-active center, an assumption which might possibly receive support from the fact that various other esterases which have a very low affinity for choline esters and for inhibitors possessing the same general configuration, are nevertheless strongly inhibited by low concentrations of DFP (10,11).

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SUMMARY

1. It has been shown that the affinity of the cholinesterases for eserine, prostigmine, and certain prostigmine analogs is decreased by increasing the concentration of electrolytes in the medium.

2. Alterations in the salt concentration fail to affect the affinity of the cholinesterases for diisopropyl fluorophosphate (DFP) appreciably, however; a possible explanation for the difference in the effect of electrolytes on the affinity of the cholinesterases for eserine and prostigmine on the one hand and for DFP on the other is discussed.

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The L-Amino Acid Oxidases of Snake Venom.

I. Prosthetic Group of the L-Amino Acid Oxidase of Moccasin Venom¹

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INTRODUCTION

Studies in this laboratory of the L-amino acid oxidases of snake venoms have shown that the oxidase from pit vipers and from a number of true vipers undergoes a spontaneous and completely reversible inactivation. In preliminary communications (1,2) it was noted that small concentrations of inorganic phosphate and of other trivalent anions greatly enhance this inactivation, whereas monovalent anions or catalytic amounts of substrates and flavin analogs prevent the inactivation. This inactivation has now been found to be completely reversible.

In order to establish the mechanism of the reversible inactivation of L-amino acid oxidase it became necessary to obtain the enzyme in homogeneous form and to establish the nature of its prosthetic group. The present paper demonstrates that the prosthetic group of the enzyme is flavin adenine dinucleotide (FAD); succeeding papers will deal with the purification of the enzyme, the kinetics of the reversible inactivation, and the mode of action of various ions in catalyzing or preventing the inactivation.

Soon after the discovery of L-amino-acid-oxidase activity in snake venoms (3), Zeller and his co-workers predicted (4,5) that the enzymes responsible for this oxidation were probably flavoenzymes on the basis

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of a rough parallelism found between the riboflavin content or yellow color, and L-amino-acid-oxidase activity of three species of venoms. The flavin nature of this oxidase was further indicated by the observation that analogs of riboflavin, which selectively inhibit yellow enzymes (6), were strongly inhibitory to the L-amino acid oxidase of all venoms tested (1). The question remained whether flavin mononucleotide, flavin adenine dinucleotide, or another derivative of riboflavin served as the prosthetic group. The recent isolation of the enzyme in this laboratory in homogeneous form, as judged by physical measurements (7), enabled us to decide the question.

EXPERIMENTAL

Apparatus

The fluorometric measurements were carried out in a Coleman photoelectric fluorometer; light absorption was measured in the Beckman spectrophotometer. Filter-paper chromatograms were developed in an apparatus similar to that described by Hotchkiss (8).

Materials

Riboflavin and cytochrome c were Wyeth products of high purity. Flavin adenine dinucleotide (FAD) was prepared from yeast by the method of Warburg and Christian (9) and triphosphopyridine nucleotide (TPN) from hog liver by the method of LePage and Mueller (10). Three preparations of flavin mononucleotide (FMN) were used; one was obtained by hydrolysis of FAD with nucleotide pyrophosphatase; the second was synthesized according to Kuhn *et al.* (11); the third one, prepared by a new method from sodium diriboflavin pyrophosphate, was the kind gift of Dr. C. W. Sondern (White Laboratories, Inc.). Adenylic acid of analytical purity was obtained from E. Bischoff and Co. and tris-(hydroxymethyl)-aminomethane (recrystallized three times) from Commercial Solvents, Inc. The split protein of D-amino acid oxidase was made by the method of Negelcin and Brömel from sheep kidneys (12) and the apoenzyme of cytochrome c reductase (TPN) from ale yeast by the method of Haas, Horecker, and Hogness (13). Adenylic deaminase was isolated from rabbit muscle (14). The nucleotide pyrophosphatase was kindly provided by Dr. A. Kornberg of the National Institutes of Health.

All venoms, except as noted, were purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, and were of uniform activity and flavin content. The only treatment these samples received prior to shipping was a brief centrifugation to remove cell debris, followed by drying in a vacuum desiccator at approximately 0°. We are thankful to Major General S. S. Sokhey, Haffkine Institute, Bombay, for kindly preparing and sending us lyophilized preparations of cobra venom (*Naia naia*), common krait venom (*Bungarus caeruleus*), and Russell's viper venom (*Vipera russelli*).

Effect of Flavin Analogs

As noted in the introduction, low concentrations of riboflavin and of its analogs inhibited the L-amino acid oxidases of various venoms. A typical experiment is summarized in Table I. It is apparent that of all the flavins and related compounds tested, riboflavin itself was the most inhibitory. A $4 \times 10^{-5} M$ solution inhibited the enzyme from moccasin

TABLE I

The Inhibition of L-Amino Acid Oxidases by Riboflavin and Related Substances

| Source of enzyme | Inhibitor | O ₂ uptake in 30 min. | | Inhibition per cent |
|------------------|---|----------------------------------|----------------|---------------------|
| | | Control | With inhibitor | |
| | | cu. mm. | cu. mm. | |
| Moccasin venom | Riboflavin $0.4 \times 10^{-3} M$ | 223.8 | 87.3 | 61 |
| Moccasin venom | Riboflavin $0.04 \times 10^{-3} M$ | 223.8 | 166.9 | 26 |
| Moccasin venom | Isoriboflavin ^a $0.4 \times 10^{-3} M$ | 223.8 | 114.5 | 49 |
| Moccasin venom | Isoriboflavin ^a $0.04 \times 10^{-3} M$ | 223.8 | 207.8 | 7 |
| Moccasin venom | Dichloroflavin ^a $0.9 \times 10^{-3} M$ | 143.0 | 129.0 | 10 |
| Moccasin venom | Atabrine ^c $2 \times 10^{-3} M$ | 159.3 | 82.5 | 48 |
| Moccasin venom | Alloxazine ^c $1 \times 10^{-3} M$ | 186.8 | 142.1 | 24 |
| Moccasin venom | Proflavine ^c $1 \times 10^{-3} M$ | 186.8 | 117.5 | 37 |
| Moccasin venom | Lumazine ^b $1 \times 10^{-3} M$ | 186.8 | 184.3 | 0 |
| Moccasin venom | Dimethyl lumazine ^b $1 \times 10^{-3} M$ | 186.8 | 191.7 | 0 |
| Cobra venom | Isoriboflavin $1 \times 10^{-3} M$ | 108.7 | 56.6 | 48 |
| Copperhead venom | Riboflavin $1 \times 10^{-3} M$ | 187.2 | 127.9 | 32 |
| Copperhead venom | Isoriboflavin $1 \times 10^{-3} M$ | 187.2 | 154.9 | 17 |

Conditions: 2 mg. dried cobra venom (*Naia naia*), 0.7 mg. dried copperhead venom, and 0.7–1.0 mg. dried moccasin venom in a final volume of 3 ml. Buffer, $8 \times 10^{-2} M$ tris-(hydroxymethyl)-aminomethane. Substrate, $7 \times 10^{-3} M$ L-leucine. Temperature, 38°C. Water-soluble inhibitors were neutralized to pH 7.2 before use; others dissolved in a small quantity of $1 \times 10^{-2} M$ NaOH immediately before use. pH = 7.2 in all vessels.

^a Dichloroflavin and isoriboflavin, both analytically pure compounds, were the generous gifts of Prof. Paul Karrer of the University of Zürich and of Dr. A. Gibson, Merck and Co., respectively.

^b We are grateful to Dr. A. Welch of the Department of Pharmacology for samples of the various pure lumazines used in this work.

^c Atabrine hydrochloride (Winthrop-Stearns, Inc.) proflavine dihydrochloride (Bios, Inc.), and alloxazine (Bios, Inc.) were used without further purification.

venom 61%; 10^{-3} *M* riboflavin inhibited 100%, and a slight inhibition (10%) could be noted even at 1×10^{-5} *M* concentration. Isoriboflavin, although toxic to microorganisms and higher animals, was somewhat less inhibitory, whereas dichloroflavin, the most toxic known analog of riboflavin, inhibited the enzyme at 9×10^{-4} *M* concentration only to a very slight extent. Atabrine, proflavine, and alloxazine were less inhibitory in the order mentioned. These compounds are not true analogs of riboflavin but are closely related structurally; atabrine is a known flavoenzyme inhibitor (15,16,17). Lumazine and dimethyl lumazine, which represent a radical departure from the isoalloxazine nucleus, failed to inhibit the enzyme at any concentration tested. Thus it appears that the closer the analog resembles the structure of the prosthetic group of the enzyme (which will be shown to be FAD), the greater the inhibitory tendency. It may be added that the ability of various riboflavin analogs to prevent the inactivation of this enzyme by inorganic phosphate approximately parallels their inhibitory power, indicating a definite order of affinity for the enzyme protein (1).

The oxidase from copperhead venom, which resembles very closely the enzyme from moccasin, and the oxidase of cobra venom, which differs markedly in the protein component from the moccasin enzyme (2,6), were also inhibited by riboflavin and isoriboflavin, but higher concentrations were required to produce the same effect.

The inhibition by these analogs is instantaneous, or nearly so; it is not prevented or reversed by the substrate or by FAD or FMN, at least in low concentrations (isoriboflavin 9×10^{-4} *M*, FAD or FMN 3×10^{-6} *M*). This is in contrast to the action of these reagents on other yellow enzymes, wherein a readily reversible competitive inhibition has been observed (6). While these observations make a displacement of the prosthetic group of the enzyme by the inhibitory analog appear unlikely, it seems nonetheless possible that these reagents combine with the enzyme protein by virtue of their similarity to the prosthetic group.

Characterization of the Prosthetic Group

All attempts to resolve the enzyme into native apoenzyme and prosthetic group failed, at least in working with crude or partly purified preparations. Attempts at resolution included prolonged dialysis at various pH values at 0°C. and 25°C., treatment with acid and $(\text{NH}_4)_2\text{SO}_4$, and incubation with the apoenzyme of D-amino acid oxidase from

kidney and with nucleotide pyrophosphatase. It was necessary therefore to purify the enzyme in order to demonstrate its flavin nature and to obtain its prosthetic group in pure form.

a) Isolation of L-Amino Acid Oxidase From Moccasin Venom

Details of the fractionation procedure and measurements of the purity of the protein will be reported in a subsequent paper in this series. The essential steps in the isolation are as follows. The bulk of inert protein is removed by heat denaturation in the presence of L-leucine (which protects the enzyme) and by adsorption on $\text{Ca}_3(\text{PO}_4)_2$ gel at pH 6.6. The enzyme is then adsorbed at its isoelectric point (pH 5.5–5.6) on $\text{Ca}_3(\text{PO}_4)_2$ gel. After preliminary washing of the gel with acetate buffer at the isoelectric point of the enzyme, the latter is eluted with 0.65 saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The enzyme is precipitated from the eluate in essentially homogeneous form by raising the $(\text{NH}_4)_2\text{SO}_4$ saturation to 0.78, at the isoelectric point of the enzyme.

b) Absorption Spectrum of Enzyme

An enzyme preparation isolated as described above, was dialyzed for 12 hr. against running distilled water in a rocking dialyzer at 5°C. One and a half milliliters of the solution was diluted with 2 ml. $1 \times 10^{-1} M$ tris-(hydroxymethyl)-aminomethane buffer, pH 7.2, and the absorption spectrum was measured at 25°C. in quartz cells of 10 mm. light path. The results are illustrated in Fig. 1 (circles). Since molecular weight measurements on the enzyme are not yet complete, optical density (D) rather than molecular extinction coefficients are plotted on the ordinate against wavelengths in millimicrons on the abscissa.³ The spectrum is typical of a flavoenzyme. Maxima were noted at 273, 389, and 465 $m\mu$, and a smaller one at 490 $m\mu$ (cf. inset); the minima were at 250, 330, and 420 $m\mu$. As in other flavoproteins (18), both the 375 $m\mu$ and the 450 $m\mu$ bands of flavins show about a 15 $m\mu$ shift toward the red, when combined with the protein part of the enzyme. The shift in the 375 $m\mu$ band is slightly greater than in other known flavoenzymes (18). The spectrum of the enzyme resembles most closely that of the "new yellow enzyme" of Haas (19) inasmuch as the 465 $m\mu$ band is slightly lower than the 389 $m\mu$ band, in contrast to other established

³ Since protein concentration was usually measured as light absorption at 280 $m\mu$ no dry weights are available for this preparation as yet.

flavoproteins, FAD, and riboflavin. If we assume, following the convention of Ball (18), that at the 465 $m\mu$ peak the L-amino acid oxidase has an absorption coefficient $\beta = 2.40 \times 10^7 \text{ cm}^2 \text{ mole}^{-1}$, then at 389 $m\mu$, $\beta = 2.50 \times 10^7 \text{ cm}^2 \text{ mole}^{-1}$. It may be added that at 275 $m\mu$ some two-thirds of the absorption is due to protein.

After measurement of the aerobic spectrum, 0.2 ml. $1 \times 10^{-1} M$ L-leucine was added to the enzyme. The yellow color disappeared immediately and the enzyme stayed reduced for a sufficient length of time

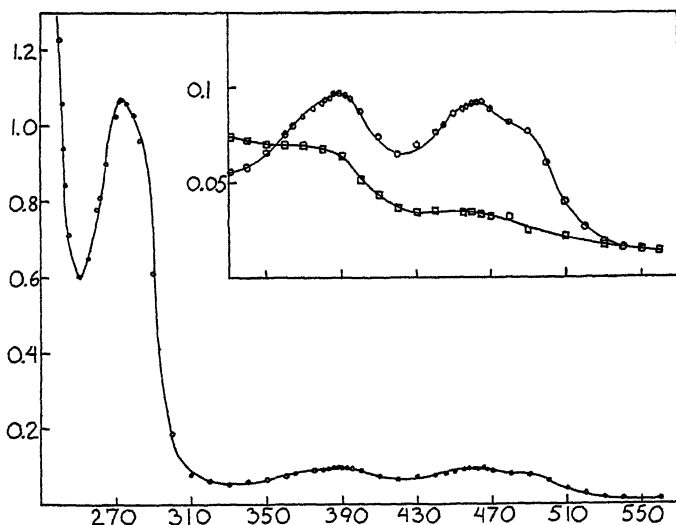


Fig. 1. Absorption spectrum of the enzyme. Abscissa, wavelength in millimicrons. Ordinate, optical density in 10-mm. cells. Circles, enzyme alone; squares (lower curve in inset), enzyme and L-leucine. The ordinate in the inset is magnified to show the peaks in the visible region. Experimental conditions in text.

to permit measurement of the spectrum of the enzyme-leucoflavin complex. The squares in Fig. 1 represent the results. For facility of comparison, the inset in Fig. 1 reproduces the visible spectrum on a larger scale, before and after reduction. The 465 $m\mu$ band disappeared completely and the 389 $m\mu$ band was largely bleached. Reduction of the enzyme with hydrosulfite and reduction with L-leucine under completely anaerobic conditions gave curves identical with this. Apparently the dehydrogenation of the flavoenzyme by the substrate is so much faster than its reoxidation by oxygen that most of the enzyme stays in the

reduced state as long as the substrate is not exhausted. As a matter of fact, even when air was bubbled through the solution at a moderate rate, much of the enzyme stayed in the reduced state. Lastly it is noted that solutions of the enzyme do not fluoresce, as is also true of other flavoproteins.

c) Preparation of the Prosthetic Group From the Purified Enzyme

The prosthetic group was split off by heat denaturation of the enzyme as described below. It was then characterized by means of its absorption spectrum, fluorescence before and after hydrolysis, by the presence of FMN, adenosine-5-phosphate, and adenine in the hydrolysis products, and by enzymatic means, using D-amino acid oxidase and cytochrome c reductase.

Five milliliters of the homogeneous enzyme solution was dialyzed for 24 hr. against distilled water. The clear solution was immersed for 2.5 min. in a boiling water bath. After cooling to 0°C., 1 ml. of 5% HPO_3 was added to complete the deproteinization, and the suspension was centrifuged for 5 min. in the cold room. The supernatant was immediately neutralized with 2 M KOH to pH 7, followed by addition of 0.05 volumes of 0.2 M tris-(hydroxymethyl)-aminomethane buffer, pH 7.2, and the yellow, fluorescent solution was diluted to 1×10^{-5} M concentration with water, as judged from the 450 m μ reading of an aliquot of the solution.

d) Absorption Spectrum

Figure 2 represents the absorption spectrum of the prosthetic group thus obtained, read in quartz cuvettes of 10 mm. light path against a blank solution containing buffer and neutralized metaphosphate in the same concentration as the unknown. The molar extinction coefficient,⁴ \mathcal{E} , is plotted on the ordinate against wavelength on the abscissa. The figures were calculated from the density readings by the use of the molar extinction coefficient of FAD at 450 m μ given by Warburg and Christian⁵ ($\mathcal{E} = 11.3 \times 10^6 \text{ cm}^2 \text{ mole}^{-1}$) (9). Readings were taken simultaneously on a 1×10^{-5} M riboflavin standard (not shown in Fig. 2). The absorption maxima were at the same points as observed with

⁴ $\mathcal{E} = \frac{1}{c} \log_{10} \frac{I_0}{I}$, where c is expressed in moles/ml., and l is the light path in cm.

⁵ Warburg states that \mathcal{E} at 450 m μ is equal for riboflavin and FAD. Actually, $\mathcal{E}_{450\text{m}\mu}$ for riboflavin is $12.2 \times 10^6 \text{ cm}^2 \text{ mole}^{-1}$.

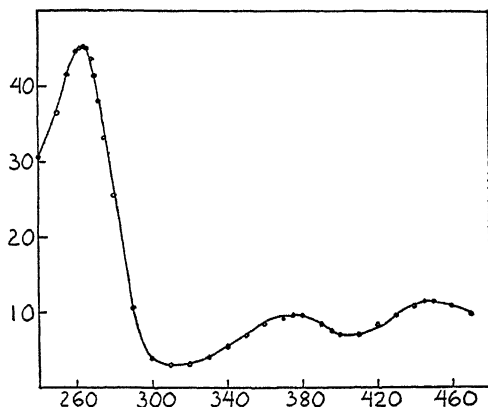


FIG. 2. Absorption spectrum of the prosthetic group. Abscissa, wavelength in millimicrons. Ordinate, molecular extinction coefficient (\mathcal{E}) $\times 10^5 \text{ cm}^2 \cdot \text{mole}^{-1}$.

authentic FAD (9); *i.e.*, at 262 $m\mu$, 375 $m\mu$, and 445–50 $m\mu$. The solution absorbed slightly less than riboflavin in the region of 350–85 $m\mu$ and slightly more than riboflavin in the region over 450 $m\mu$. Both of these observations parallel the behavior of FAD from yeast. The ratio of the 375:450 $m\mu$ peaks was about the same (1:1.2) as found by Warburg and Christian.

e) Differential Fluorometry

The fluorescence of FAD amounts to 14% of the fluorescence of an equimolar riboflavin standard; upon hydrolysis of the pyrophosphate linkage the fluorescence becomes equal to that of riboflavin (20).

Samples (0.2 ml.) of the prosthetic group preparation, $1 \times 10^{-5} M$ on the basis of its absorption at 450 $m\mu$, were hydrolyzed by means of 5% CCl_3COOH overnight at 38°C. (20); other aliquots were hydrolyzed by means of highly purified nucleotide pyrophosphatase⁶ (21) in 30 min. at 38°C. The solution was protected from light during this procedure. The reaction involved is $\text{FAD} + \text{H}_2\text{O} \rightarrow \text{FMN} + \text{adenylic acid}$. Samples of the untreated and of the hydrolyzed preparations were examined for fluorescence, with the use of an internal riboflavin standard. After readings were taken, it was ascertained that the fluorescence

⁶ Dr. A. Kornberg, National Institutes of Health, who kindly supplied this preparation, states that it had an activity of 2000 units/mg. protein. A unit is defined as one μmole of DPN split/hr. at 38°C. and pH 7.4.

TABLE II
Differential Fluorometry of Prosthetic Group

| Treatment | Riboflavin equivalent of fluorescence | | Fluorescence before hydrolysis | |
|--------------------------------------|---------------------------------------|-----------------------|--------------------------------|-----------------|
| | Calculated | Found | Calculated | Found |
| | $\mu\text{moles/ml.}$ | $\mu\text{moles/ml.}$ | <i>per cent</i> | <i>per cent</i> |
| None | 1.4×10^{-3} | 1.2×10^{-3} | 14 | 12 |
| CCl_3COOH hydrolysis | 10.0×10^{-3} | 9.9×10^{-3} | | |
| Enzymatic hydrolysis | 10.0×10^{-3} | 10.0×10^{-3} | | |

Experimental conditions: 0.1 ml. 1×10^{-5} M flavin solution and 1 ml. 1×10^{-1} M phosphate buffer, pH 7.1, in a total volume of 10 ml. Temperature, 38°C . In the acid hydrolysis experiment, 0.2 ml. 1×10^{-5} M prosthetic group plus 0.2 ml. 10% CCl_3COOH were incubated at 38°C . for 16 hr. After neutralization, an aliquot containing 1×10^{-3} μmole flavin was read against a corresponding blank under the conditions outlined above. In the enzymatic hydrolysis 5 ml. 1×10^{-5} M flavin, 0.4 ml. 2×10^{-1} M tris-(hydroxymethyl)-aminomethane, pH 7.2 and 0.3 ml. ≈ 3 units⁶ of nucleotide pyrophosphatase were incubated at 38° . Aliquots, containing 1×10^{-3} μmole total flavin, were removed periodically, and the fluorescence examined as above after dilution. Riboflavin equivalents are calculated on the basis of internal standards. Riboflavin equivalent is defined as the concentration of riboflavin which would give the same fluorescence as that observed.

was completely bleached by addition of a hydrosulfite solution. The results are summarized in Table II. Both the extent of fluorescence before hydrolysis and its increase on treatment with the specific pyrophosphatase and CCl_3COOH are in good agreement with the expected values for FAD of similar concentration.

f) Demonstration of the Presence of Adenosine-5-phosphate

Another sample of the prosthetic group was treated with nucleotide pyrophosphatase, as above, and the reaction was followed fluorometrically. When hydrolysis was complete, the resulting solution, containing equal concentrations of FMN and adenylic acid [adenosine-5-phosphate (AMP)], was 1.03×10^{-5} M, as calculated from the 450 $\mu\mu$ absorption and the fluorescence. A 2-ml. aliquot of the solution was treated with 0.4 ml. of 3×10^{-1} M succinate buffer, pH 5.9, and 0.5 ml. of 1.8 M KCl. (The high salt concentration was found necessary for deaminase activity as well as to prevent precipitation of the enzyme.) After taking initial readings in the spectrophotometer at 265 $\mu\mu$ and at 248 $\mu\mu$ against an appropriate blank, 0.1 ml. of an adenylic deam-

inase preparation was added to both. The changes in absorption involved in the enzymatic conversion of adenylic acid to inosinic acid were followed at 265 $m\mu$, and the deamination of a pure AMP standard ($1 \times 10^{-5} M$) was simultaneously observed. It has been demonstrated by Kalckar (14) that the decrease in absorption at 265 $m\mu$ as a result of enzymatic deamination under these conditions is a quantitative measure of the true adenosine-5-phosphate content.

As shown in Table III, the adenylic acid concentration calculated from the decrease in absorption at 265 $m\mu$ agreed well with the value calculated from the total flavin content, considering the small magnitude of ΔD values. It should be added that the enzymatic deamination

TABLE III

Demonstration of the Presence of Adenosine-5-phosphate by Means of Adenylic Deaminase

| Substrate | Decrease in light absorption at 265 $m\mu$ | Concentration | |
|---|--|---------------------------|----------------------|
| | | Calculated | Found |
| AMP standard | ΔD 0.0755 | M 1×10^{-5} | M |
| Prosthetic group after nucleotide pyrophosphatase treatment | 0.053 | 6.8×10^{-6} | 7.0×10^{-6} |

Experimental conditions in text. The decrease in light absorption is recorded as the difference between the ΔD value before addition of the deaminase and after 25 min., when the reaction was complete. One-cm. cells were used, at 25°C., pH 5.9. The calculated molarity is based on weight in the case of the AMP standard and on the 450- $m\mu$ absorption and fluorometric estimation in the case of the prosthetic group.

of the unknown and of the AMP standard at similar concentration followed identical rates. No change in absorption was observed at 248 $m\mu$, which is the isobestic point of adenylic and inosinic acids.

Another sample of the prosthetic group was treated with adenylic deaminase prior to the addition of nucleotide pyrophosphatase. Under these conditions no change in light absorption was observed at 265 $m\mu$. The experiments with adenylic deaminase and the differential fluorometry are offered as evidence that all of the prosthetic group is in the form of flavin dinucleotide, and that, in view of the known specificity of adenylic deaminase, adenosine-5-phosphate is a constituent part of the molecule. Supporting evidence was obtained by separation of adenine from a hydrolysate, as shown in the next section.

g) Filter Paper Chromatography of Adenine

Since the amount of material available was too small for the isolation of adenine and its identification by means of derivatives, advantage was taken of the sensitivity of the chromatographic method for the separation and identification of purines as used by Hotchkiss (8). A 1-ml. portion of the prosthetic group used in the previous experiments, $1 \times 10^{-5} M$ on the basis of light absorption and differential fluorescence, was refluxed with 2 ml. of $3 \times 10^{-1} M$ HCl for 30 min. in a boiling water bath in order to hydrolyze off the purine. The resulting solution was concentrated to dryness at low temperature *in vacuo*, and the residue was redissolved in 0.2 ml. water. This solution was then transferred to a strip of filter paper (2 cm. in width and 32 cm. long) in 0.01-ml. portions, drying each amount added under an infrared lamp. Adenine and riboflavin standards, at a range of concentrations, were chromatographed simultaneously. The solvent used consisted of 1 part of water, 1 part of diethylene glycol, and 4 parts of *n*-butanol, and the atmosphere was kept saturated with NH_4OH . After 7 hr. at $28^\circ C$. the filter papers were cut into 1-cm. strips between the solvent point and the starting point, and each strip was soaked in 0.5 ml. $1 \times 10^{-1} M$ phosphate buffer, pH 7.2, for 6 hr., with continuous shaking. After a brief centrifugation, the absorption of each extract was examined in microcells of 10 mm. light path in the spectrophotometer. On those extracts which absorbed significantly at the peak of adenine ($260 m\mu$), the spectrum was also determined over the range of 235–275 $m\mu$, in steps of 5 $m\mu$. The R_F value for the adenine standards was = distance traveled by adenine/distance traveled by solvent front = 13.8 cm./20.1 cm. = 0.68. The result for the prosthetic group sample was R_F = 12.4 cm./18.3 cm. = 0.68. The chromatographic peak obtained was very sharp, as determined by light absorption at 260 $m\mu$, and the spectrum corresponded to that of adenine. Quantitative estimation was somewhat difficult because of the known interference by breakdown products of riboflavin; however, the value estimated was within 10% of the expected amount of adenine.

h) Estimation of FMN by Means of Cytochrome Reductase

If the prosthetic group of L-amino acid oxidase is indeed FAD, it should be possible to demonstrate that it is fully active in replacing the prosthetic group of D-amino acid oxidase, but incapable of serving as

the prosthetic group of cytochrome c reductase (TPN), unless hydrolyzed previously, in accordance with the known prosthetic group requirements of these enzymes. The latter point was checked by splitting yeast cytochrome c reductase [purity, 0.32 (13)] with acid in the presence of $(\text{NH}_4)_2\text{SO}_4$, redissolving the apoenzyme in phosphate buffer, and assaying its activity in the presence of graded concentrations of FMN, according to the method of Haas, Horecker, and Hogness (13). Using three different FMN preparations as mentioned under *Materials*, the saturation curve of the enzyme was determined spectrophotometrically, and the FMN concentration of unknown samples was read from this standard curve. After appropriate corrections for the blanks given by the cytochrome reductase present in the *Zwischenferment* preparation as well as by the nonenzymatic reduction of cytochrome c (22), a $1.66 \times 10^{-7} M$ solution of the prosthetic group (as determined from light absorption at $450 m\mu$) activated cytochrome reductase to the same extent as did $5 \times 10^{-9} M$ FMN. Thus only some 3% of the flavin present behaved as the mononucleotide in this specific enzymatic test. This could be considered negligible, in view of the fact that this amount of breakdown of FAD to FMN could be expected during the boiling and acid treatment in the course of isolation.

In contrast to this minute amount of FMN before hydrolysis, other samples of the prosthetic group hydrolyzed by nucleotide pyrophosphatase as outlined above, behaved as pure FMN and gave the correct saturation curve with the apoenzyme of cytochrome reductase.

i) Test with D-Amino Acid Oxidase Apoenzyme

Since FMN has only a small fraction of the activity of FAD in converting the apoenzyme of D-amino acid oxidase to the active holoenzyme, it was of interest to compare the activity of the prosthetic group we have isolated with authentic FAD in this test, as final proof of the identity of the compound.

D-Amino acid oxidase activity was measured manometrically in the usual way (9). Two sets of saturation curves were determined under the conditions recommended by Warburg and Christian (9), and several additional curves were obtained under the slightly different experimental conditions used by Hellerman *et al.* (17). The results were calculated from a plot of the total flavin concentration against the O_2 consumption, by taking the flavin concentration corresponding to half-

maximal velocity. The results were also checked by the method of Lineweaver and Burk (23)⁷. The apparent K_M observed with the prosthetic group isolated from L-amino acid oxidase ($2 \times 10^{-7} M$) was in good agreement with the value found with authentic FAD samples in this laboratory, and with the data similarly calculated from the figures given by Hellerman *et al.* (2.0 – $2.1 \times 10^{-7} M$) who have made a thorough study of the dissociation constants of D-amino acid oxidase. The maximal velocity observed with the enzyme, using the prosthetic group sample, was identical with that given by pure FAD as the source of the prosthetic group. Since contamination of an FAD preparation with FMN or other flavins usually depresses the maximal velocity and increases the apparent K_M value (6), it was concluded that other flavins were absent from the preparation. Inasmuch as FAD is the only compound known to activate D-amino acid oxidase to a maximal extent, these experiments, together with the evidence cited in the other sections, demonstrate that the prosthetic group of L-amino acid oxidase is indeed FAD.

DISCUSSION

In his review of the enzymes of snake venom, Zeller (5) cited several criteria by which the L-amino acid oxidases of snake venoms differ from the enzyme isolated from rat kidney by Green's group (24) and from the L-amino acid oxidase of *Proteus vulgaris* (25). To these we may now add the fact that the L-amino acid oxidase of moccasin venom contains FAD as its prosthetic group, in contrast to the oxidase from rat kidney, which is reported to require FMN, whereas the oxidase from *Proteus* does not seem to be a flavoenzyme.

In view of the high FAD content of moccasin venom, it was of interest to determine whether all the flavin in the crude venom is in the form of the dinucleotide and whether all the flavin present can be accounted for in terms of the L-amino acid oxidase content of the crude venom. Several batches of moccasin venom were heat-inactivated or depro-

⁷ The apparent K_M thus observed is not a true dissociation constant, as shown by Hellerman *et al.* (17), since a considerable part of the FAD is bound to the protein and since saturation of the enzyme with respect to substrate must also be taken into account in calculating the dissociation constant, K_{FS} . The true dissociation constant was not calculated in these experiments, since for the purpose of identification of the compound with FAD it seemed adequate to demonstrate that with the same enzyme preparation, under identical conditions, the compound and FAD gave identical apparent K_M values.

teinized by mild treatment⁸ under conditions which minimized enzymatic or thermal breakdown of FAD, and the total flavin content of the deproteinized solution was estimated from the light absorption at $450\text{ m}\mu$ to be $4 \times 10^{-1}\text{ }\mu\text{mole/g.}$ of dried venom. This figure was in good agreement with the FAD content of the venoms as estimated with D-amino acid oxidase. Thus all the flavin in the venom can be accounted for as FAD. In the purification of the enzyme, the flavin follows the enzyme activity in a regular fashion, and the yield of flavin parallels the yield of enzyme. Thus, in one large isolation experiment, the homogeneous L-amino acid oxidase was obtained in 43% yield, and the FAD, split off as described above, and its concentration estimated by means of spectrophotometry, fluorometry, and the D-amino acid oxidase test, also gave a 43% yield, as compared with the flavin contained in the starting material. Thus it seems probable that all the flavin present in the crude venom is part of the amino acid oxidase and that no other flavoenzyme is present.

So far, our isolation work has been limited to the oxidase of moccasin venom, but measurements of the FAD content of Russell's viper venom and of krait venom indicate that the L-amino acid oxidase of these venoms is also an FAD enzyme, although these species represent members of different zoological families.

It should be added that when crude snake venoms are heat-inactivated in order to liberate the protein-bound FAD, the dinucleotide breaks down very extensively; under similar conditions it is stable in animal tissues or in the pure L-amino acid oxidase. Thus, heated solutions of crude venom may give variable results in the activation of D-amino acid oxidase apoenzyme, with lower saturation velocities and larger K_M values than are obtained with pure FAD. Fluorometric determinations and assays with cytochrome reductase also attest to the fact that nearly all of the FAD may be broken down during the heat treatment. The extent of breakdown depends on the pH and on the rate of heating; it seems greatest when solutions of the venom are treated with ethanol at room temperature; the latter effectively denatures the oxidase and liberates the protein-bound flavin. It seems quite probable that the breakdown is enzymatic in nature and that the enzyme responsible is rather stable to heat and alcohol treatment, but cannot act on FAD which is bound to a native enzyme. Experiments are in progress to determine whether it is identical with the "adenosine triphosphatase"

⁸ Two min. at 98–99°C. in *dilute* solution at pH 5.5.

reported by Zeller (26) to be present in all snake venoms, since the specificity of the latter enzyme is as yet unknown.

The high FAD content of snake venoms and the apparent absence of other flavins offers an opportunity for the preparation of small amounts of FAD of high purity with relative ease, compared with the laborious procedure involved in its isolation from yeast or liver. Although this can be accomplished in good yield and in a few hours working time by isolation of the L-amino acid oxidase and subsequent deproteinization, we are working on a simplified method for the isolation of FAD from snake venoms.

SUMMARY

1. The L-amino acid oxidase of moccasin venom has been isolated in homogeneous state. It shows an absorption spectrum typical of flavo-proteins. The absorption bands in the visible region are bleached by the substrate even under aerobic conditions.

2. The prosthetic group of the enzyme has been split off and identified as FAD by its absorption spectrum, fluorescence before and after hydrolysis, as well as by the demonstration of the presence of adenosine-5-phosphate and of adenine among the products of hydrolysis. The prosthetic group quantitatively replaces FAD in the D-amino acid oxidase test, but activates the apoenzyme of cytochrome c reductase from yeast only after hydrolysis by nucleotide pyrophosphatase.

3. The riboflavin content of the crude moccasin venom has been found to be 4×10^{-1} μ moles/g. and all of it could be accounted for as FAD bound to L-amino acid oxidase. Other venoms have also been found to be rich sources of FAD.

4. Riboflavin and certain of its analogs have been shown to inhibit the enzyme at low concentrations, but the inhibition is not a reversible competition with the prosthetic group.

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Absence of Growth-Inhibiting Activity in Trypsin Inhibitor from Egg White

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INTRODUCTION

The trypsin inhibitor in egg white has been prepared in purified form by Lineweaver and Murray (1) with *in vitro* activities of the same order of magnitude as the most highly purified fractions of trypsin inhibitor from soybeans or lima beans. Demonstration in rats, mice, and chicks of growth inhibition by various soybean and lima bean fractions possessing high *in vitro* trypsin-inhibiting activity has been made in several laboratories (2-6). Borchers, Ackerson, and Mussehl (7) alone have prepared from soybeans a trypsin inhibitor which had no measurable growth-inhibiting activity. However, as they pointed out at that time, a growth inhibitor free of trypsin inhibitor had not been prepared from soybeans or similar sources. That is, crude preparations containing both factors have not been successfully separated into two mutually exclusive factors. Since at least three distinct trypsin inhibitors have been reported for soybeans (8), the question of whether some trypsin inhibitors have growth-inhibiting activity remains unanswered. The present report contributes additional information to this problem by showing that the well-characterized trypsin inhibitor from egg white (ovomucoid), like the one isolated by Borchers *et al.*, has no growth-inhibiting activity for rats. Whereas this is of fundamental and supplemental interest in regard to possible growth-inhibiting characteristics of trypsin inhibitors, it is of practical interest also because of the nutritional importance of eggs and the high content of trypsin inhibitor which they contain.

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EXPERIMENTAL RESULTS AND DISCUSSION

Egg white trypsin-inhibitor preparations³ were fed to young rats (70-90 g.) on a purified adequate diet containing casein as the base protein, according to the experimental procedure described in detail previously (2,3). Average feed consumption and weight gains over a 5-day period are presented in the following table.

TABLE I
*Effect of Trypsin-Inhibiting Fraction from Egg White on Weight Gains
of Rats on an Adequate Diet*
(14.5% total crude protein in diet; 10 rats per group)

| Group | Protein supplement in addition to casein ^a | Feed consumption/ rat/day | Average gain/ rat/day | Gain/g. of nitrogen eaten |
|---------|---|------------------------------|--------------------------|------------------------------|
| Expt. A | | g. | g. | g. |
| 1 | None | 12.7 | 4.3 | 14.5 |
| 2 | 0.5% of egg-white prepn. No. 1, containing 9 trypsin inhibitor units/mg. ^b | 12.1 | 4.1 | 14.5 |
| 3 | 0.5% of egg-white prepn. No. 1, inactivated by heat; about 1 trypsin-inhibitor unit/mg. | 11.7 | 4.0 | 14.5 |
| 4 | 0.9% of lima-bean fraction, containing 4-5 trypsin-inhibitor units/mg. | 11.1 | 1.7 | 6.6 |
| Expt. B | | | | |
| 1 | 0.5% of egg-white prepn. No. 2, containing 9 trypsin-inhibitor units/mg. | 11.1 | 4.7 | 19.3 |
| 2 | 0.75% of egg-white prepn. No. 2 | 11.1 | 4.8 | 19.7 |
| 3 | 1.00% of egg-white prepn. No. 2 | 10.6 | 4.6 | 19.8 |
| 4 | 1.0% of egg-white prepn. No. 2, inactivated by heat, about 1 trypsin-inhibitor unit/mg. | 10.9 | 5.3 | 22.2 |

^a Casein protein decreased by amount of other protein present.

^b 1 Trypsin-inhibitor unit, as measured and defined by Lineweaver and Murray (1) is the amount of activity in 1 mg. of standard dried egg white.

No appreciable inhibition of growth was observed at any of the levels of egg-white trypsin inhibitor fed. A reduction of more than 50% in

³ The large amounts of pure egg-white trypsin inhibitor were generously supplied by Dr. Hans Lineweaver and Mr. C. W. Murray. We are also indebted to them and to Mr. Ross S. Bean for the *in vitro* trypsin-inhibitor assays of the various preparations.

weight gained was obtained when 0.9% of the ration consisted of a lima bean fraction, which contributed about the same number of *in vitro* trypsin-inhibitor units as the 0.5% level of egg white preparation No. 1. In a third feeding experiment, a 2.5% level of the egg white trypsin inhibitor also failed to cause a growth depression.

It has been shown (2) that crude bean trypsin inhibitors cause less growth inhibition when the base diet protein is casein than when it is soybean, and cause still less growth inhibition when the base protein is egg white. In the present experiment, feeding the purified egg-white trypsin inhibitor in a casein diet eliminated the possibility that any growth-inhibiting activity might be counteracted by other protein constituents in egg white.

Recent results (5,6,9) indicate that inhibition of protein hydrolysis in the digestive tract is not the principal cause of growth inhibition by bean protein fractions. That is, bean growth-inhibitor preparations are active in animals fed diets of hydrolyzed proteins. However, since all growth-inhibiting preparations from beans so far tested have trypsin-inhibiting activity, the possibility exists that some trypsin inhibitors have growth-inhibiting properties for some reason other than the inhibition of proteolysis. This possibility has now been eliminated for two of the many known trypsin inhibitors, namely, the inhibitor from egg white and one of the trypsin inhibitors from soybeans. Negative results with the egg-white trypsin inhibitor do not necessarily prove that an effective trypsin inhibitor in the intestinal tract would have no effect on growth. The trypsin inhibitor may be inactivated before it reaches that part of the digestive tract where it might inhibit proteolysis. The observation that egg-white trypsin inhibitor is inactivated slowly by pepsin tends to support this possibility (10).

SUMMARY

The purified trypsin inhibitor from egg white has been shown to have no growth inhibiting activity when fed to rats at levels as high as 2.5% of the diet.

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Catalytic Decomposition of Hydroxylamine by Hemoglobin

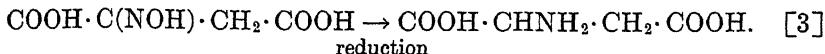
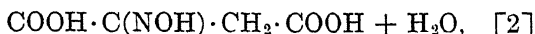
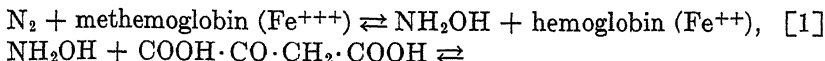
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INTRODUCTION

Kubo (1) discovered that hemoglobin (Hb) exists as a red pigment in the nodules of legumes. Keilin and Wang (2) confirmed this discovery, showing that the pigment of soybean nodules is a hemoglobin with a characteristic absorption spectrum. Neither the plant cells alone, nor the *Rhizobia* grown separately, synthesize hemoglobin. The facts point to the possibility that in the root nodule, hemoglobin itself, or the processes connected with its synthesis, may be directly connected with nitrogen fixation. On the other hand, hemoglobin may act only indirectly by securing in the root nodule optimal conditions for oxidative processes with which nitrogen fixation may be associated. Virtanen and Laine (3) [see also Virtanen (4,5)] have found methemoglobin in root nodules and consider that an equilibrium exists there between hemoglobin and methemoglobin. As a result of experiments on the excretion of nitrogen products by legumes they have made a hypothesis expressed by the following equations² relating nitrogen fixation to valency changes in hemoglobin.



Keilin and Smith (6) deny, however, that methemoglobin exists as a

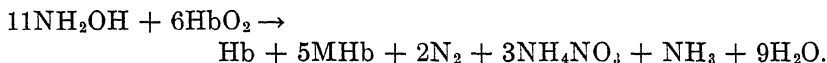
¹ Canada Packers Research Fellow, McGill University, Montreal.

² They make the proviso that the hydroxylamine mentioned in the first equation need not appear directly from molecular nitrogen but possibly only after a number of intermediate stages.

normal constituent of the root nodule and do not support the view that nitrogen fixation involves valency changes in hemoglobin.

Damodaran and Varma (7) have reported the isolation of hydroxylamine from the nodules of six different legumes.

It has been known for many years that reactions take place between hemoglobin and hydroxylamine. Letsche (8) and Heubner (9) found that oxyhemoglobin (HbO_2) is transformed into methemoglobin by hydroxylamine, nitrogen being formed. Two molecules of hydroxylamine were oxidized by one of oxyhemoglobin. Lipschitz and Weber (10) found that hydroxylamine is rapidly decomposed to yield nitrogen and ammonia under the catalytic influence of hemoglobin and postulated that methemoglobin was involved in the reaction. They found that the velocity of the reaction depended on the quantity of hemoglobin present, a maximum being reached when the ratio of hemoglobin to hydroxylamine is 1:2. Roche (11) considered that hydroxylamine breaks down in the presence of oxyhemoglobin according to the scheme:



It is known that hydroxylamine forms a complex with a hemoprotein such as catalase [Keilin and Hartree (12)], inhibiting its catalytic activity. The hydroxylamine, however, is not decomposed in this association.

The present work was undertaken to throw more light on the mode of breakdown of hydroxylamine by hemoglobin and to obtain more information on the relation between hydroxylamine and the enzyme systems that are affected by it.

EXPERIMENTAL

Preparations

Lysed red blood cells. Human blood, freshly collected in citrate-glucose anticoagulant, was centrifuged, and the cells separated from the plasma. The cells were washed four times by suspending in cold 0.9% NaCl solution and centrifuging 5 min. at 2000 r.p.m. After the final washing, the cells were lysed by adding a carefully measured volume to a known quantity of cold distilled water (usually 4 vols. water to one vol. cells). The hemoglobin solution so obtained was kept in the cold.

Methemoglobin solution. To a portion of hemoglobin solution was added sufficient $\text{K}_3\text{Fe}(\text{CN})_6$ solution to convert all the hemoglobin to methemoglobin. Excess ferri-cyanide, and the ferrocyanide produced, were removed by dialysis against cold, running, tap water for several hours.

Native globin was prepared by the method of Jope (13), and was converted into

methemoglobin by the addition of hemin in 0.1 *N* NaOH. (One mole hemin was added to 1 equivalent of globin, the equivalent weight being taken as 17,000.)

Hydroxylamine solution. Hydroxylamine hydrochloride was weighed, dissolved in distilled water, and carefully neutralized with dilute NaOH solution. Hydroxylamine solutions were freshly made up before each experiment, as were all reagents with the exception of the hemoglobin solution.

Sodium azide solution. Reagent grade NaN₃ was weighed, and dissolved in distilled water.

Cyanide solution was made up from reagent grade NaCN, dissolved in distilled water and neutralized with dilute HCl immediately before use.

Cysteine solution was made up from cysteine hydrochloride, the pH of the solution being adjusted to 7.0 with dilute NaOH immediately before use.

Ascorbic acid solution was made by dissolving crystalline ascorbic acid in distilled water and exactly neutralizing with dilute NaOH immediately before use.

Procedure

Experiments were carried out manometrically using Warburg constant-volume manometers at 37°, the pH of the reaction mixture being maintained at pH 7.4 by means of 0.1 *M* sodium phosphate buffer. The hydroxylamine solution was added to the hemoglobin solution at zero time by tipping from the side arm of the Warburg vessel, after attainment of temperature equilibrium. Readings were begun 3–5 min. after the addition. In all experiments, except where indicated, the gas phase was nitrogen.

After the reaction had proceeded for the desired length of time, the manometers were taken quickly from the bath, the vessels removed, and their contents tipped at once into 10% trichloroacetic acid solution. The precipitated protein was removed by filtration, and the filtrate, after neutralizing to pH 7.0, was used for analysis.

Analytical Methods

Ammonia. Ammonia was estimated by the method of Markham (14). Hydroxylamine, which was found to interfere with the estimation, was destroyed by carrying out the steam distillation of ammonia in the presence of MnO₂.

Hydroxylamine was estimated manometrically by tipping 0.4 ml. suspension of 1 g. MnO₂ (British Drug Houses) in 10 ml. sodium phosphate buffer (pH 7.4) from the side tube of a Warburg manometric vessel into the main vessel, which contained the sample of neutral filtrate, and measuring the volume of N₂O evolved at 37°C. The method was based on the technique of Mann and Quastel (15) in their studies of manganese metabolism in soil. A variation of the method has already been used by Jacquemain and Galliot (16). Calculations were based on the equation



and the accuracy of the method permitted the estimation of hydroxylamine with an experimental error not exceeding $\pm 1.5\%$.

Sodium azide was estimated manometrically, the method depending on the fact that azide is converted to nitrogen by iodine solution in the presence of a trace of sodium thiosulfate. Hydroxylamine, which interferes with the estimations, was removed by treating the sample of filtrate with MnO_2 before proceeding with the azide estimation. 0.4 ml. 0.2 *N* iodine (in KI) solution was placed in the side tube of a Warburg manometric vessel and tipped into the main vessel containing sodium azide solution and two drops of 0.1 *N* sodium thiosulfate solution. The volume of nitrogen produced at 37° was measured in the conventional manner. The following Table I shows the calculated and experimental yields of N_2 found with known quantities of azide. Calculations were based on the equation



The experimental yields of N_2 approached the theoretical the smaller the quantity of azide used. Since the values given in Table I were found to be consistently obtained for the given concentrations of azide, a calibration curve, relating nitrogen evolved to azide initially present, was constructed. This was used in later work involving azide estimations.

TABLE I
Estimation of Sodium Azide

| Sodium Azide mmoles $\times 10^{-3}$ | Nitrogen (found) mmoles $\times 10^{-3}$ | Nitrogen (calculated) mmoles $\times 10^{-3}$ |
|---|---|--|
| 5.13 | 6.56 | 7.69 |
| 3.85 | 5.18 | 5.77 |
| 2.56 | 3.44 | 3.85 |
| 1.28 | 1.83 | 1.92 |

Sodium azide estimation, in the presence of hydroxylamine, was carried out after decomposing the hydroxylamine with MnO_2 . The procedure was to place the azide-hydroxylamine mixture in the main compartment of a Warburg manometer vessel, add 0.4 ml. MnO_2 suspension, as previously prepared, followed by two drops $\text{Na}_2\text{S}_2\text{O}_4$, and place 0.4 ml. iodine solution in the side tube. After temperature equilibration, the taps of the apparatus were closed and the iodine tipped in. The volume of nitrogen evolved was measured in the conventional manner. This method permitted an accurate estimation of sodium azide in the presence of hydroxylamine, the experimental error involved not exceeding $\pm 1.0\%$.

EXPERIMENTAL RESULTS

Decomposition of Hydroxylamine by Hemoglobin under Anaerobic Conditions

When 0.2 ml. of a neutral solution of hydroxylamine is tipped, in an atmosphere of nitrogen, from the side tube of a Warburg manometer

vessel into the main compartment containing a solution of hemoglobin, gas evolution takes place at once. The rate of evolution of the gas at 37° is measured in the conventional manner. Analysis of the contents of the manometer vessel at various intervals of time shows that ammonia is a product of the reaction, but there is no indication of nitrate formation [see Roche (11)].

Analyses were made, from time to time, of the hydroxylamine and ammonia present in the reaction mixture. The manometric readings gave measures of amounts of nitrogen formed. A balance sheet of the nitrogen distribution was drawn up and the results are given in Table II.

TABLE II
Breakdown of Hydroxylamine by Hemoglobin
All quantities given in terms of mmoles

| Time of expt. | Experimental | | | | NH ₃ equivalent to N ₂ formed (Eq. 6) | NH ₂ OH equivalent to N ₂ formed (Eq. 6) | NH ₃ formed minus N ₂ equivalent | NH ₂ OH utilized minus N ₂ equivalent | Ratio: NH ₃ /NH ₂ OH |
|---------------|--------------------------------------|-----------------------------|-----------------------|------------------------|---|--|--|---|--|
| | NH ₂ OH initially present | NH ₂ OH utilized | N ₂ formed | NH ₃ formed | | | | | |
| <i>min.</i> | | | | | | | | | |
| 5 | 0.4 | 0.042 | 0.005 | 0.032 | 0.005 | 0.015 | 0.027 | 0.027 | 1.00 |
| 15 | 0.4 | 0.056 | 0.01 | 0.035 | 0.01 | 0.03 | 0.025 | 0.026 | 0.96 |
| 30 | 0.4 | 0.067 | 0.0135 | 0.037 | 0.0135 | 0.041 | 0.0234 | 0.026 | 0.90 |
| 90 | 0.4 | 0.119 | 0.031 | 0.057 | 0.031 | 0.093 | 0.026 | 0.026 | 1.00 |
| 165 | 0.4 | 0.129 | 0.030 | 0.066 | 0.030 | 0.090 | 0.036 | 0.039 | 0.92 |
| 165 | 0.1 | 0.067 | 0.0165 | 0.034 | 0.0165 | 0.0495 | 0.0175 | 0.0175 | 1.00 |
| 165 | 0.1 | 0.078 | 0.0205 | 0.036 | 0.0205 | 0.0615 | 0.0155 | 0.0165 | 0.94 |

It will be seen that the breakdown of the hydroxylamine may be wholly accounted for, within experimental error, by the formation of nitrogen and ammonia. It will also be seen that initially the rate of ammonia production exceeds that of nitrogen evolution.

Effects of Varying Hemoglobin Concentrations on the Rate of Anaerobic Decomposition of Hydroxylamine

The rates of nitrogen evolution at 37° from 0.133 *M* hydroxylamine in phosphate buffer pH 7.4 and in the presence of various concentrations of lysed red cells are shown in Fig. 1. The rates of nitrogen evolution are initially rapid, but diminish to approximately steady values after the first 30 min. Moreover, it is evident from Fig. 1, that the rates are

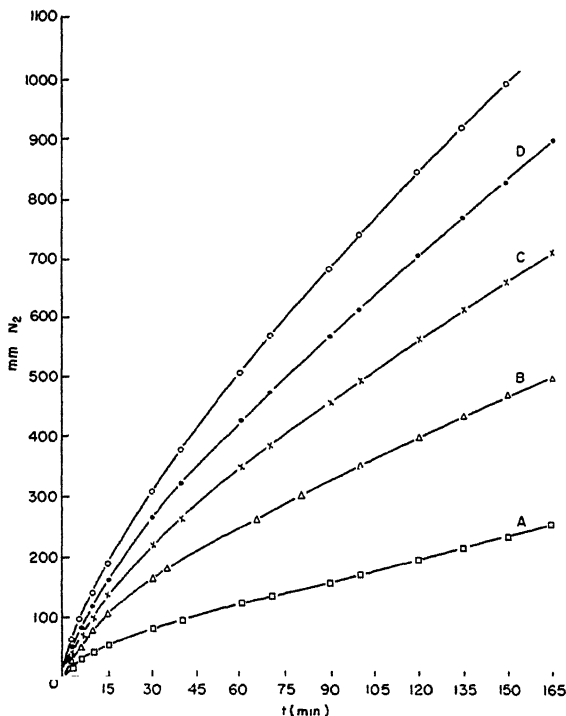


FIG. 1. The effect of hemoglobin concentration on the rate of decomposition of hydroxylamine (0.133 M) at pH 7.4. Total volume 3.2 ml. In nitrogen. A 0.2 ml. lysed red blood cells (r.b.c.). (1:4); B 0.4 ml. lysed r.b.c. (1:4); C 0.6 ml. lysed r.b.c. (1:4); D 0.8 ml. lysed r.b.c. (1:4); E 1.0 ml. lysed r.b.c. (1:4).

directly proportional to the hemoglobin concentrations when these are low, although there is a departure from direct proportionality at high hemoglobin concentrations.

Substantially the same quantitative results were obtained when the reactions were carried out in vessels containing yellow phosphorus to absorb any oxygen that might be liberated from oxyhemoglobin present.

Effects of Varying Hydroxylamine Concentrations on the Rates of Nitrogen Evolution

The effects of varying the hydroxylamine concentration on the rates of nitrogen evolution in the presence of 1.0 ml. lysed human red blood cells (lysed by adding 1 volume of cells to 4 volumes of water) at pH

7.4 are shown in Fig. 2. The rates increase with increase of hydroxylamine concentration, and are directly proportional at relatively low hydroxylamine concentrations.

Rates of Ammonia and Nitrogen Formation During the Anaerobic Decomposition of Hydroxylamine in the Presence of Hemoglobin

Results given in Fig. 3 show the relative rates of ammonia and nitrogen production during the breakdown of hydroxylamine by 1.0 ml. lysed human red blood cells (1 volume of cells + 4 volumes of water) at pH 7.4. It is noteworthy that there is a rapid formation of ammonia immediately following the addition of hydroxylamine to the hemo-

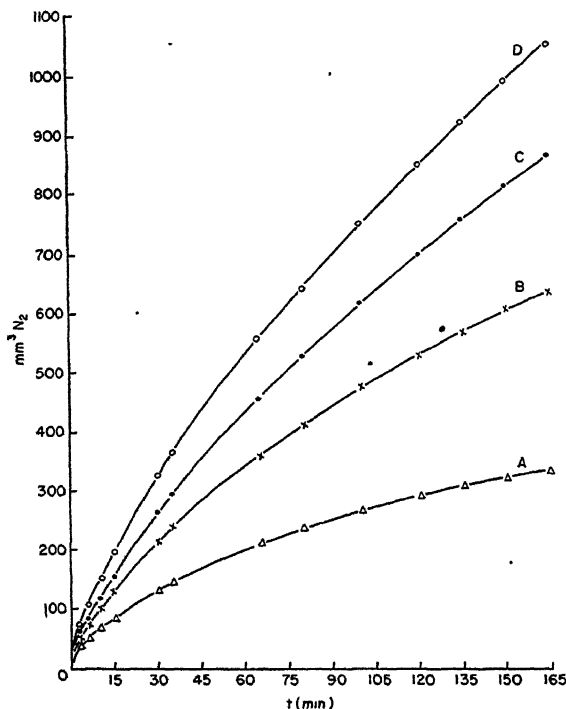


FIG. 2. The effect of hydroxylamine concentration on the rate of its decomposition in the presence of 1.0 ml. lysed red blood cells (1:4), at pH 7.4; total volume 3.2 ml., in nitrogen. A 0.034 M NH_2OH ; B 0.067 M NH_2OH ; C 0.100 M NH_2OH ; D 0.133 M NH_2OH .

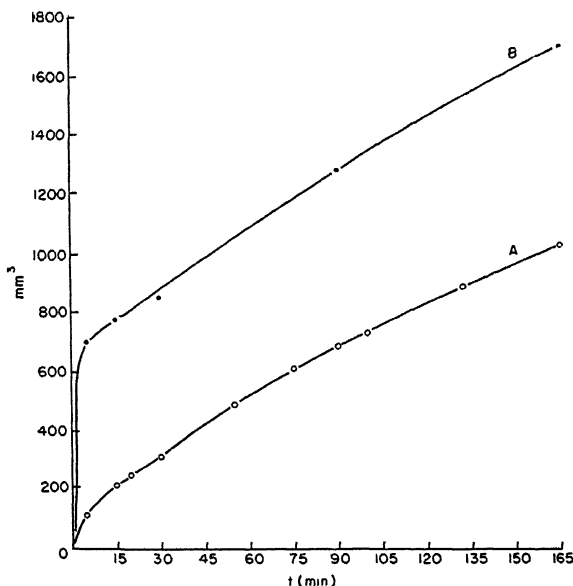
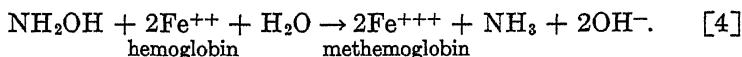


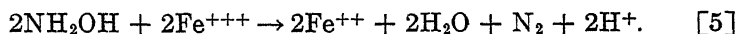
FIG. 3. Rates of ammonia and nitrogen production by the decomposition of hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4 Total volume 3.2 ml. in nitrogen. A, N₂, B, NH₃.

globin solution. This is succeeded by a slower steady rate of ammonia formation which is equal to that of the nitrogen evolution.

This result seems to be most satisfactorily explained by assuming that the first reaction, on adding hydroxylamine to hemoglobin, is a reduction of the former to ammonia and an oxidation of the latter to methemoglobin, thus:



When the methemoglobin is formed, a second reaction takes place whereby nitrogen is evolved with reduction of the methemoglobin to hemoglobin, thus:



The steady, equal, rates of ammonia and nitrogen formation are held to be due to the combined operation of reactions [4] and [5]. If these are added, the following equation is obtained:



Such a reaction constitutes a dismutation of hydroxylamine induced by the catalytic action of hemoglobin. In such a system, hemoglobin acts as an enzyme, accomplishing its activity by reversible oxidation and reduction, in a manner similar to that held to take place with catalase or cytochrome oxidase.

Evidence in favor of this hypothesis may be secured by analysis of the values given in Table II. Examination of this table shows that if there are subtracted from the values of NH_2OH utilized and NH_3 formed, the values of NH_2OH and NH_3 calculated according to Eq. 6 from the values of N_2 formed, amounts of NH_2OH and NH_3 remain whose ratio is approximately unity. Moreover these amounts do not increase markedly during the first 90 min. of the experiment. This conclusion is to be expected if the first reaction undergone by hydroxylamine is a direct reduction to NH_3 according to Eq. 4 and if this is followed by a catalytic dismutation of the hydroxylamine according to Eq. 6. Thus although the net values of nitrogen production from hydroxylamine are lower than the theoretical 0.33 moles N_2 for each mole of hydroxylamine decomposed (according to Eq. 6), the values are in strict accordance with dismutation when allowance has been made for the direct initial reduction of hydroxylamine to ammonia.

Effect of Heat Treatment of Hemoglobin

If the proteins of the lysed red blood cells are denatured by heating, catalytic decomposition of hydroxylamine ceases or is very greatly diminished.

Effect of Air on the Decomposition of Hydroxylamine by Hemoglobin

When hydroxylamine is added to a solution of lysed red blood cells in the presence of air instead of nitrogen, the evolution of gas takes place at a lower rate. Thus whereas 0.2 ml. 2 *M* NH_2OH yielded 695 cu. mm. nitrogen in the presence of 1 ml. lysed red blood cells in 165 min. at pH 7.4 in an atmosphere of nitrogen, it yielded 468 cu. mm. nitrogen under the same conditions in an atmosphere of air. It is possible that the gas liberated contains a little oxygen arising from oxyhemoglobin present. However, estimations showed that 1 ml. of lysed red blood cells contained approximately 40 mg. of hemoglobin, and the maximum volume of oxygen which could be liberated from this amount

of hemoglobin, were it all in the form of oxyhemoglobin, would be approximately 42 cu. mm.

*Action of Cyanide on the Decomposition of Hydroxylamine
in the Presence of Hemoglobin*

The presence of 0.01 *M* cyanide almost completely suppresses the evolution of nitrogen from hydroxylamine in the presence of hemoglobin at pH 7.4. This result is shown in Fig. 4. This effect is probably due to the formation of cyanmethemoglobin, which is catalytically inert.

*Effects of Cysteine and Ascorbic Acid on the Decomposition
of Hydroxylamine in the Presence of Hemoglobin*

The addition of cysteine, or of ascorbic acid, or of a mixture of these substances, brings about a large increase in the rate of ammonia pro-

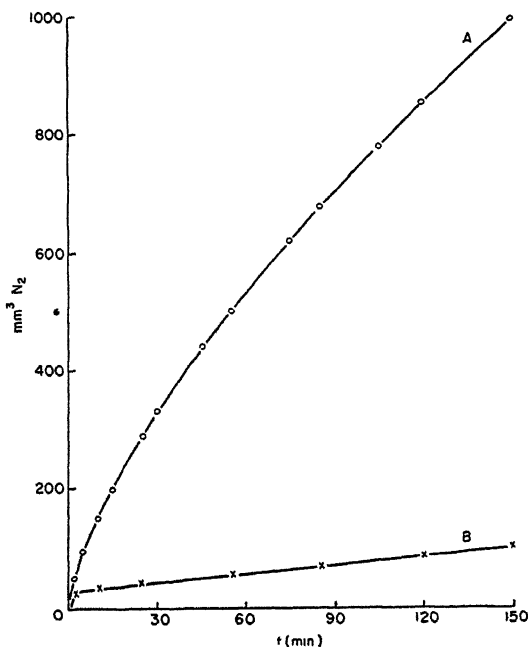


FIG. 4. The effect of cyanide on nitrogen formation from hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4. Total vol. 3.2 ml., in nitrogen. A, no CN^- present; B, 0.01 *M* CN^- .

duction from hydroxylamine in the presence of hemoglobin with a corresponding decrease in the rate of nitrogen formation. Typical curves showing the effects of cysteine and ascorbic acid, at pH 7.4, on the rates of nitrogen formation from hydroxylamine in the presence of hemoglobin are given in Figs. 5 and 6. Results indicating the relative amounts

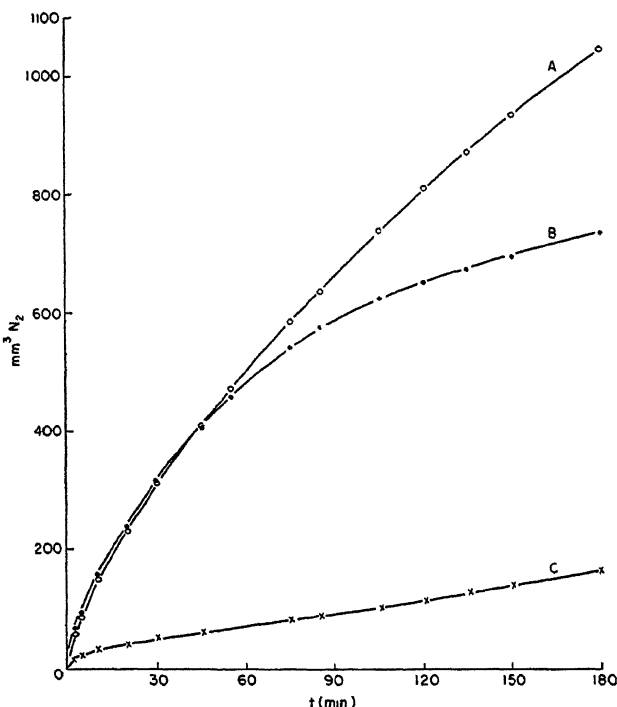


FIG. 5. The effect of cysteine and ascorbic acid on nitrogen formation from hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4. Total vol. 3.2 ml., in nitrogen. *A*, control. No cysteine or ascorbic acid present; *B*, 33 mg. cysteine; *C*, 50 mg. ascorbic acid.

of nitrogen and ammonia produced in the reaction are shown in Table III. The amounts of ammonia are expressed in cu. mm. (1 g.-mole \equiv 22.4 l.).

It is evident from an examination of the results given in Table III that both cysteine and ascorbic acid bring about a considerable reduction of hydroxylamine to ammonia in the presence of hemoglobin. The

hemoglobin appears to act catalytically, for there is much less reduction in its absence. The velocity of nitrogen formation in the presence of hemoglobin is very greatly suppressed, especially in the presence of ascorbic acid.

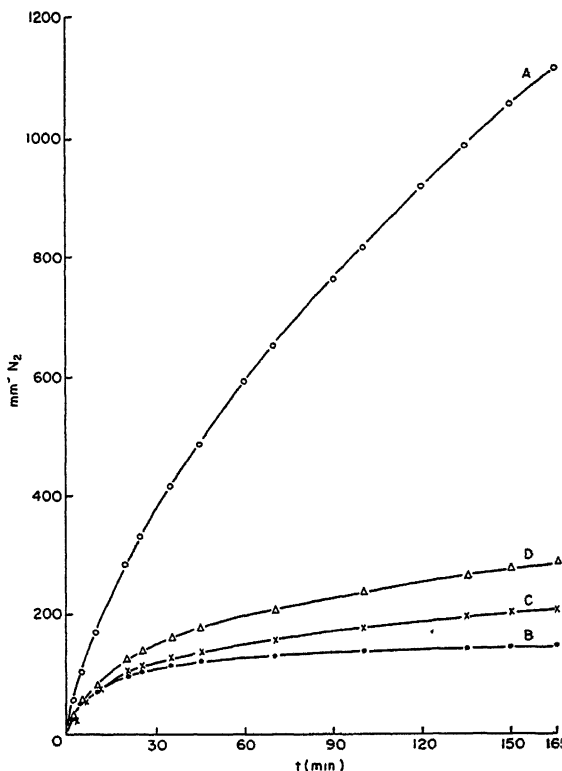
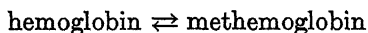


FIG. 6. The effect of cysteine and ascorbic acid on nitrogen formation from hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4), at pH 7.4. Total vol. 3.2 ml. In nitrogen. A, control; B, 33 mg. cysteine + 50 mg. ascorbic acid; C, 33 mg. cysteine + 20 mg. ascorbic acid, D, 33 mg. cysteine + 10 mg. ascorbic acid.

The mechanism of this action is most clearly understood on the assumption that both cysteine and ascorbic acid, and particularly the latter, are more vigorous reducers of methemoglobin than is hydroxylamine. The result is that, in their presence, the equilibrium



is being constantly shifted to the left so that increased ammonia production, in accordance with Eq. 4, and decreased nitrogen formation ensue.

The observation is of interest physiologically, for it is apparent that under conditions in the living cell when hydroxylamine and hemoglobin are together, the presence of ascorbic acid, or of a suitable thiol compound, will tend to stimulate the formation of ammonia and to suppress

TABLE III

Effects of Cysteine and Ascorbic Acid on Hydroxylamine Breakdown

Decomposition of hydroxylamine (3 ml. 0.133 *M*) in the presence of 1 ml. lysed human red blood cells (1 cells:4 water) and in the presence of cysteine hydrochloride (30 mg. neutralized) or ascorbic acid (50 mg. neutralized) at pH 7.4. N₂. 37°. Experimental time = 165 min. Initial concentration NH₂OH = 0.133 *M*.

| Lysed red cells | Cysteine HCl | Ascorbic acid | N ₂ evolved | NH ₃ produced |
|-----------------|--------------|---------------|------------------------|--------------------------|
| <i>ml.</i> | <i>mg.</i> | <i>mg.</i> | <i>cu. mm.</i> | <i>cu. mm.</i> |
| 1.0 | — | — | 1045 | 1840 |
| 1.0 | 30 | — | 741 | 3900 |
| 1.0 | — | 50 | 163 | 4590 |
| — | 30 | — | — | 530 |
| — | — | 50 | — | 610 |
| 1.0 | — | — | 1121 | 1935 |
| 1.0 | 30 | 50 | 146 | 5520 |
| 1.0 | 30 | 20 | 206 | 5250 |
| 1.0 | 30 | 10 | 286 | 5100 |
| — | 30 | 50 | — | 450 |

the evolution of nitrogen. Doubtless, part of the initial direct reduction of hydroxylamine to ammonia by lysed red blood cells is due to the ascorbic acid, and possibly glutathione, present.

The Effects of Sodium Azide on the Decomposition of Hydroxylamine in the Presence of Hemoglobin

The presence of sodium azide inhibits the breakdown of hydroxylamine in the presence of hemoglobin. The magnitude of the inhibition, however, instead of increasing with increase of azide concentration, actually decreases as the azide concentration is increased. Typical results are shown in Fig. 7. In fact, at an azide concentration of 0.1 *M*, the production of nitrogen is markedly increased above the normal rate

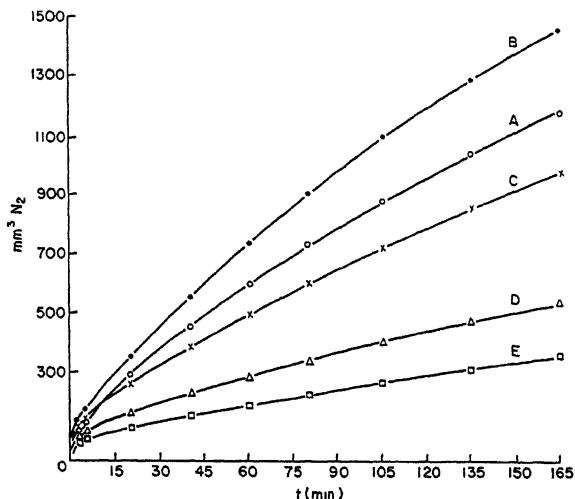


FIG. 7. The effect of sodium azide on the decomposition of hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4. Total vol. 3.2 ml. In nitrogen. A, control; B, 0.1 *M* NaN₃; C, 0.06 *M* NaN₃; D, 0.03 *M* NaN₃; E, 0.01 *M*. NaN₃.

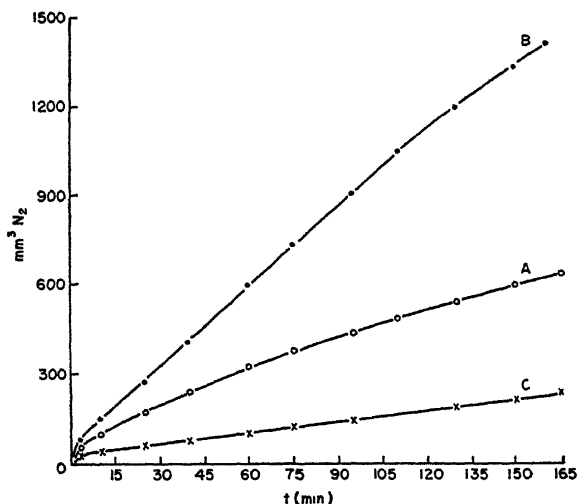


FIG. 8. The effect of sodium azide on the decomposition of hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4. Total vol. 3.2 ml. In nitrogen. A, control; B, 0.1 *M* NaN₃; C, 0.01 *M* NaN₃.

of formation (see Fig. 8). Moreover, it is clear that during the reaction part of the azide undergoes chemical change. When a balance sheet is constructed between the amounts of hydroxylamine and azide decomposed (as determined by analysis) and the amounts of nitrogen and ammonia formed, it is evident that a discrepancy is present. Typical values are shown in Table IV.

Except with low concentrations of azide there is failure to obtain complete recovery of the nitrogen. There is evidence to indicate that the discrepancy is due to combination between azide and methemoglobin (17), this complex being precipitated by trichloroacetic acid so that the azide so combined is lost in the analytical procedure.

TABLE IV

Effect of Sodium Azide on the Decomposition of Hydroxylamine

Balance sheet relating to breakdown of hydroxylamine by hemoglobin (1 ml. lysed red blood cells^a) in presence of sodium azide at pH 7.4. N₂. 37°. Experimental time = 165 min.

| N added | | | N found as | | | | | NH ₂ OH nitrogen utilized | Excess of N(N ₂ +NH ₃) over NH ₂ OH nitrogen utilized |
|---------------------------------------|-------------------------------------|-------|---------------------------------|-------------------------------|---------------------------|--------------------------|-------|--|--|
| As NH ₂ OH initially | As NaN ₃ initially | Total | Remaining NH ₂ OH | Remaining NaN ₃ | NH ₄ formed | N ₂ formed | Total | | |
| mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| 5.6 | — | 5.60 | 3.93 | — | 0.80 | 0.87 | 5.6 | 1.67 | 0.0 |
| 5.6 | 12.6 | 18.20 | 3.85 | 8.70 | 0.69 | 1.84 | 15.1 | 1.75 | 0.78 |
| 5.6 | 7.6 | 13.20 | 4.50 | 5.40 | 0.51 | 1.22 | 11.6 | 1.10 | 0.63 |
| 5.6 | 3.8 | 9.40 | 4.84 | 2.20 | 0.34 | 0.67 | 8.1 | 0.76 | 0.25 |
| 5.6 | 1.3 | 6.90 | 5.06 | 0.97 | 0.31 | 0.44 | 6.8 | 0.54 | 0.21 |

^a One ml. cells lysed with 4 ml. water.

It is obvious, however, that azide enters into the reaction as the amount of nitrogen gas produced considerably exceeds that expected from the loss of hydroxylamine alone. This is seen in the experimental results shown in Table IV, from which it will be observed that the amount of nitrogen contained in the products N₂ + NH₃ exceeds that of the nitrogen of the hydroxylamine, which direct analysis had shown to be utilized. Since no component, other than azide, is capable of giving rise to the extra nitrogen, it is reasonable to assume that azide itself undergoes decomposition in the presence of hemoglobin and hydroxylamine. Both the latter factors must be involved, since experiment shows

TABLE V

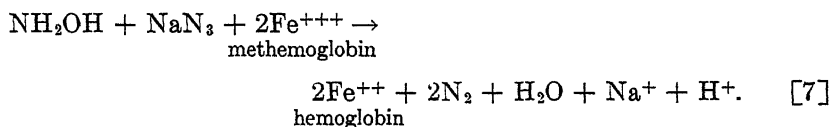
Effects of Sodium Azide on Breakdown of Hydroxylamine

Effects of addition of sodium azide on the breakdown of hydroxylamine in presence of hemoglobin (1 ml. lysed human red blood cells, 1 cells-4 water) at pH 7.4. 37°. Nitrogen. Experimental time = 165 min.

| NH ₂ OH, initially | NaN ₃ initially | NH ₂ OH recovered | NH ₂ OH utilized | NH ₃ formed | NH ₂ OH utilized minus NH ₃ equiv | N ₂ formed | Ratio NH ₂ OH utilized -NH ₃ equiv./ N ₂ formed |
|----------------------------------|-------------------------------|---------------------------------|--------------------------------|---------------------------|--|--------------------------|---|
| ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | ×10 ⁻⁵ mole | |
| 40 | Nil | 24.08 | 15.92 | 8.0 | 7.92 | 4.00 | 1.98 |
| 40 | Nil | 22.79 | 17.21 | 7.59 | 9.62 | 4.64 | 2.07 |
| 40 | Nil | 28.06 | 11.94 | 5.70 | 6.24 | 3.11 | 2.00 |
| 40 | 3.0 | 35.45 | 4.55 | 2.21 | 2.34 | 1.57 | 1.49 |
| 40 | 9.0 | 34.55 | 5.45 | 2.41 | 3.04 | 2.39 | 1.27 |
| 40 | 18.0 | 32.12 | 7.88 | 3.59 | 4.29 | 4.36 | 0.98 |
| 40 | 30.0 | 27.73 | 12.27 | 7.83 | 4.44 | 6.32 | 0.70 |
| 10 | 30.0 | 3.64 | 6.36 | 3.77 | 2.59 | 4.25 | 0.61 |

that azide does not decompose in the presence of hemoglobin or methemoglobin alone, nor in the presence of hydroxylamine alone.

A reasonable view is that the following reaction takes place:



If this view³ is correct it would be expected that in the presence of high azide concentrations the ratio of moles NH₂OH utilized minus NH₃ equivalent/moles nitrogen formed would approximate to the value 1: 2. This, in fact tends to occur, as may be seen from Table V.

*Effect of Azide on Cyanide Inhibition of Hydroxylamine
Breakdown by Hemoglobin*

In the presence of sodium azide, the addition of cyanide does not bring about an inhibition of the rate of evolution of nitrogen from hydroxylamine in the presence of hemoglobin. Results illustrating this

³ See also Foulkes and Lemberg (18) on the possibility of azide breakdown in the presence of catalase and hydrogen peroxide.

fact are shown in Fig. 9. Doubtless the lack of inhibitory action of cyanide is due to its inability to dissociate the azide-methemoglobin complex.

Effect of Hemin on the Anaerobic Breakdown of Hydroxylamine

Hemin itself can catalyze the anaerobic decomposition of hydroxylamine at 37°C., but its catalytic power is much less than that of hemoglobin. In the presence of sodium azide (0.1 *M*) it fails to show the increased nitrogen evolution exhibited by the hydroxylamine-azide-hemoglobin system. However, in the presence of reducing agents such

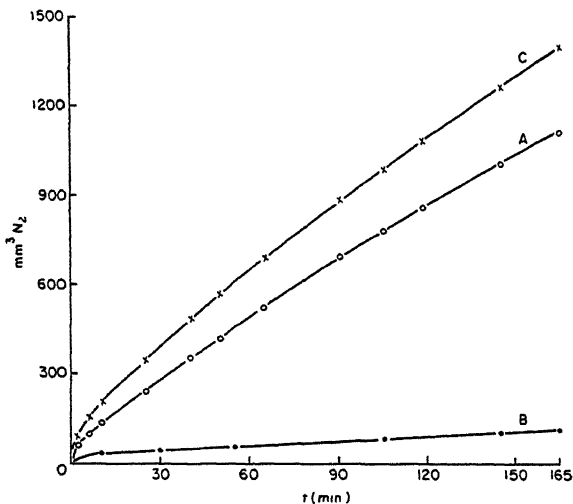


FIG 9. The effect of cyanide and azide on the decomposition of hydroxylamine (0.133 *M*) in the presence of hemoglobin (1 ml. lysed r.b.c. 1:4) at pH 7.4. Total vol. 3.2 ml. In nitrogen. A, control; B 0.01 *M* CN⁻; C, 0.01 *M* CN⁻ + 0.1 *M* NaN₃.

as cysteine and ascorbic acid, hemin proves to be an effective reducer of hydroxylamine. Table VI gives results illustrating these facts.

The quantity of ammonia formed as compared with the volume of nitrogen formed is unexpectedly high in the hemin-hydroxylamine system. This suggests that the activity of hemin may not be merely quantitatively less than that of hemoglobin, but that the mechanism of its reaction with hydroxylamine may differ from that of the hydroxylamine-hemoglobin reaction.

It seems possible that hemin enters into the ammonia-forming reaction [4] as effectively as hemoglobin; but that it is much less effective in the nitrogen-forming reaction [5].

However, the data available do not permit the drawing of any definite conclusions on this point.

*The Effect of Various Heme-Protein Complexes
on Hydroxylamine Breakdown*

An attempt was made to determine whether any specificity was associated with the protein moiety of hemoglobin in its decomposition of hydroxylamine. Several heme-protein complexes were prepared and tested to see if they were capable of catalyzing the decomposition of

TABLE VI

Breakdown of Hydroxylamine by Hemin

Decomposition of hydroxylamine at pH 7.4 in presence of hemin and lysed red blood cells. 37°. N₂. Experimental time = 165 min. Concentration of NH₂OH, 0.133 M.

| Hemin | Lysed red blood cells (1.4) | Concn. of Na.N ₃ | Cysteine | Ascorbic acid | Nitrogen formed | Ammonia formed |
|------------|-----------------------------------|--------------------------------|------------|------------------|--------------------|-------------------|
| <i>mg.</i> | <i>ml.</i> | <i>M</i> | <i>mg.</i> | <i>mg.</i> | <i>cu. mm.</i> | <i>cu. mm.</i> |
| — | 1.0 | — | — | — | 1025 | 1700 |
| 1.0 | — | — | — | — | 190.0 | 700 |
| 1.0 | — | 0.10 | — | — | 176.6 | 700 |
| 1.0 | — | — | 30 | — | 128.0 | 1980 |
| 1.0 | — | — | — | 50 | 93.8 | 2055 |
| 1.0 | — | — | 30 | 50 | 41.0 | 5250 |

hydroxylamine. The results obtained indicate that specificity is involved. The only hemin compound tested which showed any increased activity when compared with hemin was the one prepared from hemin and native globin. This preparation was shown, spectrophotometrically, to be identical with the hemoglobin of red blood cells. The results obtained with these preparations are shown in Figs. 10 and 11.

Examination of these results shows that the catalytic power of hemin itself on the decomposition of hydroxylamine is decreased by the preparations⁴ of serum albumin and serum globulin used in this way.

⁴The preparations were purified crystalline substances kindly presented to us by Dr. G. Perlmann.

A preparation of modified human globin also inhibited the normal catalytic activity of hemin. Native globin, however, prepared from human erythrocytes by the method of Joep, on addition to hemin increased its catalytic activity over 200%.

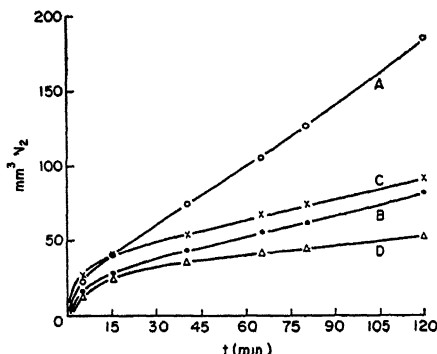


FIG. 10. The decomposition of hydroxylamine (0.133 *M*) in the presence of various heme-protein complexes at pH 7.4. Total vol. 3.2 ml. In nitrogen. A, 1.0 mg. hemin; B, 1.0 mg. hemin + 30 mg. serum albumin; C, 1.0 mg. hemin + 66 mg. serum globulin; D, 1.0 mg. hemin + 32 mg. modified human globin.

This observation proves that hemoglobin, synthesized from its constituents hemin and globin, can accomplish the catalytic breakdown of hydroxylamine.

Spectrophotometric Data

Spectrophotometric analyses support the conclusions that have been derived from the manometric and chemical data. It may be readily shown that identical absorption spectra are obtained by adding hydroxylamine solution to methemoglobin and hemoglobin solutions, or to a mixture of hemoglobin and methemoglobin.

It may also be observed that the spectrum of hemoglobin is not appreciably altered by the addition of hydroxylamine when either cysteine or ascorbic acid is present (at pH 7.4). This supports the conclusion that both cysteine and ascorbic acid tend to keep hemoglobin in its reduced form even in the presence of excess hydroxylamine.

If the hydroxylamine-hemoglobin-cyanide system (as in Fig. 4) is examined spectrophotometrically, the absorption spectrum obtained is identical with that of cyanmethemoglobin. This supports the view that cyanide inhibits hydroxylamine breakdown by immobilizing the meth-

moglobin and that it does not interfere with the oxidation of hemoglobin to methemoglobin by hydroxylamine with the accompanying production of ammonia.

It may also be shown that the spectrum obtained on adding hydroxylamine to hemoglobin in the presence of azide is identical with that of the methemoglobin-azide complex. Azide therefore does not suppress the oxidation of hemoglobin to methemoglobin by hydroxylamine.

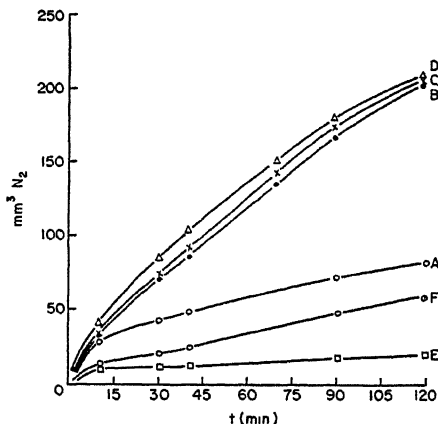


FIG. 11. The decomposition of hydroxylamine (0.133 *M*) in the presence of hemin and of synthetic hemoglobin at pH 7.4. Total vol. 3.2 ml. In nitrogen. *A*, 0.5 mg. hemin; *B*, 0.5 mg. hemin + 1.0 ml. globin solution; *C*, 0.5 mg. hemin + 1.5 ml. globin solution; *D*, 0.5 mg. hemin + 2.0 ml. globin solution; *E*, 1.5 ml. globin solution; *F*, control (NH_2OH and phosphate buffer only).

Finally, in the hydroxylamine-hemoglobin system containing cyanide (0.01 *M*) and sodium azide (0.01 *M*), the absorption spectrum seen is that of the azide-methemoglobin complex. This provides evidence that sodium azide prevents the cyanide-inhibition of this system by preventing the formation of catalytically-inert cyanmethemoglobin.

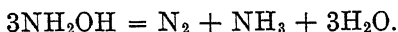
ACKNOWLEDGMENT

Our thanks are due to the National Cancer Institute of Canada for a grant in aid of a program of work, of which the material presented in this paper represents a part.

SUMMARY

1. The process of anaerobic breakdown of hydroxylamine to nitrogen and ammonia by hemoglobin has been investigated. Two reactions

appear to be involved: (1) a reduction of hydroxylamine to ammonia with oxidation of hemoglobin to methemoglobin and (2) an oxidation of hydroxylamine to nitrogen with reduction of methemoglobin to hemoglobin. On the basis of these two reactions which take place simultaneously, the kinetics and quantitative aspects of anaerobic breakdown of hydroxylamine may be satisfactorily interpreted. Hemoglobin acts, in fact, as an enzyme bringing about the dismutation of hydroxylamine according to the following equation:



2. The rates of anaerobic breakdown of hydroxylamine are directly proportional to the hemoglobin concentrations when these are low. There is a departure from direct proportionality at high hemoglobin concentrations. The rates of anaerobic breakdown are also proportional to the hydroxylamine concentration when this is low.

3. Heat treatment of hemoglobin greatly diminishes its power to catalyze the breakdown of hydroxylamine.

4. The presence of cyanide (0.01 *M*) almost completely suppresses the catalytic power of hemoglobin in the presence of hydroxylamine probably by forming catalytically-inactive cyanmethemoglobin.

5. The presence of air apparently diminishes the catalytic power of hemoglobin.

6. The addition of cysteine or of ascorbic acid brings about a large increase in the rate of ammonia production from hydroxylamine in the presence of hemoglobin with a corresponding decrease in the rate of nitrogen formation. Hemoglobin catalyzes the reduction of hydroxylamine to ammonia by cysteine or ascorbic acid.

7. Sodium azide inhibits the breakdown of hydroxylamine in the presence of hemoglobin, but azide, itself, appears to undergo breakdown in the presence of hemoglobin and hydroxylamine with evolution of nitrogen. Sodium azide may be estimated manometrically.

8. In the presence of sodium azide the addition of cyanide does not bring about an inhibition of the rate of nitrogen evolution from hydroxylamine in the presence of hemoglobin.

9. Hemin, itself, catalyzes anaerobic breakdown of hydroxylamine, but its activity as far as nitrogen formation is concerned is very much less than that of an equivalent quantity of hemoglobin.

10. This catalytic power of hemin is greatly increased by the addition of native globin, presumably because of hemoglobin production. It is decreased by the addition of other proteins investigated.

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I. *Lycopersicon* Selections Containing a High Content of Carotenes and Colorless Polyenes.¹ II. The Mechanism of Carotene Biosynthesis

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INTRODUCTION

For several years one of the objectives of our work has been the development of tomato selections of a high content of each of the carotenes and colorless polyenes reported in previous papers (1,2). Such selections would, of course, prove to be excellent sources for the isolation of uncharacterized carotenes and colorless polyenes not found in abundance elsewhere. In addition, if selections homozygous for each carotene and colorless polyene could be developed such selections could be used in studies on the inheritance of carotenes and colorless polyenes. The results of such studies would have importance from the standpoint of chemical genetics and might also supply evidence on the pathway of carotene biosynthesis.

The development of tomato selections homozygous for a high β -carotene content has been reported (3). Studies on the inheritance of β -carotene have also been reported (4). The present paper reports on the development of tomato selections containing relatively large quantities of other carotenes and colorless polyenes.

Experimental Tomatoes

Commercial garden and canning tomato varieties are members of the species *Lycopersicon esculentum* L. This species also occurs wild or escaped in many subtropical or tropical regions, and these forms have a fruit size ranging from 3 to 175 g. Fruit color is usually red, but yellow, orange, and nearly white forms occur. The red color of

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these fruits is due to lycopene. A closely related species *L. pimpinellifolium* (Jusl.) Mill. has red fruits of 0.6–2.0 g. size. *L. hirsutum*, Humb. and Bompl., a so-called "green-fruited" species, has fruits of 1.0–5.0 g. size. Its fruits are almost devoid of carotenoids and on ripening are greenish white to brown in color. Practically all of the color of these fruits is traceable to the chlorophylls, β -carotene, and anthocyanins.

Accessions of each of the above species were obtained through the courtesy of the Foreign Plant Introduction office of the U. S. Department of Agriculture. Each accession carries a P. I. number.

Most of the selections reported in this paper have been derived from advanced generations of backcrosses of several *Lycopersicon esculentum* \times *Lycopersicon hirsutum* hybrids with the *Lycopersicon esculentum* parent. A system of inbreeding with chemical analysis of progeny in each generation has been followed. At present, crosses are being made between selections high in content of a particular compound in an effort to further concentrate genetic factors for each compound under study.

EXPERIMENTAL METHODS

Chemical methods of analysis involved chromatography and spectrophotometry. In the case of tomatoes containing one or more of the compounds, phytoene,⁴ phytofluene, and ζ -carotene light-absorption readings were made on hexane extracts of the tomatoes at 2850, 3480, or 4000 Å, respectively. Hexane extracts were obtained by the method reported by Zscheile and Porter (5). Ten- to 20-ml. aliquots of those samples having the greatest absorbancy at the above wavelengths were then chromatographed on columns of ignited MgO-Super Cel (1:1 mixture) (6). Development of the column was effected with 10% of acetone in hexane. The compound or compounds of importance in each sample were collected as each passed from the column. All of the fraction preceding phytofluene was collected for a determination of phytoene content. Phytofluene was collected as the fluorescent band adsorbing below α -carotene. After removal of the acetone from each hexane solution with two water washes, light-absorption readings were made at the proper wavelength. Light-absorption curves were obtained also for the chromatographically-isolated polyene or polyenes of those samples containing the largest quantities of each of the above compounds.

In the few analyses made for the tetrahydrophytoene (7) content of tomato selections, the eluate preceding phytofluene in the above chromatographic separations was evaporated⁵ to dryness under vacuum. The residue was then dissolved in purified iso-octane and light-absorption readings were made at 2200 and 2850 Å. One-seventh⁶ of the log value obtained at 2850 Å was subtracted from the log value obtained at 2200 Å. The resulting value was then used in the calculations of tetrahydrophytoene content.

⁴ The name phytoene is given to a compound reported by Porter and Zscheile (2). It has previously been called a colorless, nonfluorescent polyene.

⁵ Commercial hexane does not transmit light of wavelength less than 2700 Å (8). Purified hexane or iso-octane will transmit light of 2000 Å or greater.

⁶ The ratio of light absorption by phytoene at 2200 Å to that at 2850 Å is 1:7 (9). More than traces of light-absorbing compounds other than phytoene and tetrahydrophytoene have not been observed in this fraction.

In the case of tomatoes containing relatively large quantities of carotenes other than γ -carotene, aliquots of hexane extracts of the tomatoes were chromatographed on MgO-Super Cel columns (1:1 mixture), and the chromatograms were developed with 10% of acetone in hexane. Extraction and phasic purification of the carotenes preliminary to chromatographic separation were made by the method previously described (5). Since large numbers of samples were analyzed, the band width of each pigment was measured, and those samples of widest band width of a particular carotene were saved. Finally a second aliquot of 15–25 ml. of extract of each of the best samples was chromatographed and the band or bands desired were scraped from the column. α -Carotene was collected as it passed through the column. The pigment of each band scraped from the column was eluted with 10% of ethanol in hexane. Alcohol was removed from the hexane by washing twice with distilled water. Quantitative light-absorption determinations were made on each pigment eluted from a chromatographic column and qualitative light-absorption curves were obtained for the pigment of those samples containing the highest content of each carotene.

The following wavelengths were used in making light-absorption readings and the following specific absorption coefficients were used in making calculations.

| | λ | Sp. α | | λ | Sp. α |
|----------------------------|-----------|--------------|-------------------------|-----------|--------------|
| Lycopene (10) | 5020 | 320 | ξ -Carotene (12) | 4000 | 220 |
| γ -Carotene (10) | 4600 | 270 | β -Carotene (13) | 4780 | 228 |
| δ -Carotene (11) | 4560 | 314 | α -Carotene (13) | 4460 | 275 |
| Prolycopene (10) | 4400 | 188 | Phytofluene (6) | 3480 | 150 |
| Protetrahydrolycopene (10) | 4300 | 156 | Phytoene (9) | 2850 | 140 |
| | | | Tetrahydrophytoene(14) | 2200 | 30 |

RESULTS

Lycopene

Commercial tomato varieties such as Rutgers or Indiana Baltimore contain approximately 90–120 μ g. of lycopene/g. of fruit. Some selections of *L. pimpinellifolium* contain a much higher content of lycopene. Thus selections from P. I. 126951, 126953, and 127833 contained between 300–400 μ g. of lycopene/g. of fruit. Since in the progeny of these stocks, lycopene constitutes 95–100% of the carotenes found, it can be assumed that these stocks are homozygous for the major factors for lycopene synthesis.

In addition to the above selections a few selections of advanced generations of the cross Indiana Baltimore \times *L. pimpinellifolium* P. I. 126093 (a selection of relatively high lycopene content) have been obtained which have commercial fruit size and contain 200 μ g. of lycopene/g. of fruit. Also, a mutant of the commercial variety, Rutgers,

has been found which averages approximately 150 $\mu\text{g.}$ of lycopene/g. of fruit.

β -Carotene

The work from this Station on selection for high β -carotene content and the inheritance of this pigment and lycopene has been reported in previous publications (3,4,15). Selections containing as high as 120 $\mu\text{g.}$ of β -carotene/g. of fruit have been derived from advanced generations of the cross Indiana Baltimore $\times F_1$ (Rutgers $\times L. hirsutum$ P. I. 126445). Large-fruited types containing up to 90 $\mu\text{g.}$ of β -carotene/g. of fruit (15 times the quantity of commercial varieties) have been developed by crossing the above selections with Rutgers and then selfing successive generations. Since selections containing 95–100% of the total carotenes as β -carotene breed true, they are considered homozygous for the major factors for the formation of β -carotene. These selections are therefore acceptable for studies on the inheritance of β -carotene.

γ -Carotene

An F_5 selection containing 11.0 $\mu\text{g.}$ of γ -carotene/g. of fruit has been obtained from the cross Selection 4079–6003⁷ \times (Marglobe Supreme $\times L. hirsutum$ P. I. 127827). Other selections with somewhat different parentage and containing almost as high a γ -carotene content have also been made. All of the selections have P. I. 127827 as a common parent. γ -Carotene has been observed in many red-fruited varieties previously, but rarely is it present to the extent of 5 $\mu\text{g.}/\text{g.}$ of fruit.

γ -Carotene comprises only 10–20% of the carotenes of the above selections. Other carotenes present include lycopene, β - and δ -carotene.

δ -Carotene

An F_3 selection of the same parentage as that which produced selections of a high γ -carotene content was crossed with the F_1 of Indiana Baltimore $\times L. pimpinellifolium$ P. I. 126951. F_3 selections from this cross have contained as much as 40 $\mu\text{g.}$ of δ -carotene/g. of fruit. Other selections of somewhat lower δ -carotene content have also been obtained. While the parentage of the latter selections varies somewhat from that of the selection containing the largest quantity of δ -carotene, all of the selections have P. I. 127827 as a common parent.

⁷ Selection 4079–6003 is from the cross F_3 [Indiana Baltimore $\times F_1$ (Rutgers $\times L. hirsutum$ P. I. 126445)].

δ -Carotene comprises 40–60% of the carotenes of the above selections. Lycopene, γ - and β -carotene are also present.

*Prolycopene and Protetrahydrolycopene*⁸

These compounds, as might be expected from their structural similarity, occur together in several tomato selections and varieties. They were first noted in the variety Tangerine (18) and they are now known to occur in the variety, Golden Jubilee (1). Several selections have been obtained from advanced generations of the backcross of [Pritchard \times Pritchard $\times F_1$ (Pritchard $\times L. hirsutum$ P. I. 127827)] which also contain relatively large quantities of prolycopene and protetrahydrolycopene. An F_6 selection of this backcross contained 26 $\mu\text{g.}$ of prolycopene and 16 $\mu\text{g.}$ of protetrahydrolycopene/g. of fruit.

Prolycopene and protetrahydrolycopene comprise approximately 30–50% of the carotenes and colorless polyenes of the above selections. Other compounds present include ζ -carotene, phytofluene, phytoene, β -carotene, and tetrahydrolycopene.

ζ -Carotene, Phytofluene, and Phytoene

Selections containing a high content of any one of these three compounds have been derived from the same crosses, and, with few exceptions, those selections containing a large amount of one compound also contain considerable quantities of the other two. F_3 selections from the backcross [Pan American $\times F_1$ (Pan American $\times L. hirsutum$ P. I. 126446)] have proven to be unusually high and relatively uniform in content of these compounds. A selection containing approximately 61, 30, and 38 $\mu\text{g.}$ of ζ -carotene, phytofluene, and phytoene, respectively, had the highest content of each of these compounds. Other individual selections have analyzed as high as 85 $\mu\text{g.}$ of ζ -carotene, 30 $\mu\text{g.}$ of phytofluene and 43 $\mu\text{g.}$ of phytoene/g. of fruit. Each compound comprises 40–70% of the colorless polyene and carotene content in the selections highest in that compound. Prolycopene, protetrahydrolycopene, tetrahydrolycopene, and β -carotene are also found in these selections.

ζ -Carotene and phytoene have never been reported in commercial tomato varieties. It is now known, however, that they occur along with

⁸ This compound was formerly called all-*cis*-lycopene (10), poly-*cis*- ψ -carotene (16) and unidentified I (1). Recently evidence has been obtained through isomerization studies that this compound is a poly-*cis*-tetrahydrolycopene (17).

phytofluene in varieties such as Golden Jubilee and Tangerine and in commercial tomato paste. Phytofluene has been isolated from commercial tomato paste by Zechmeister and Sandoval (19).

α -Carotene

α -Carotene is present in trace quantities in commercial tomato varieties. However, some selections derived from the same cross that produced tomatoes of high γ - and δ -carotene content have contained as much as 9.0 $\mu\text{g.}$ of α -carotene. The pedigree for the cross producing selections of a high α -carotene content is given under the paragraph on γ -carotene.

α -Carotene comprises 10–20% of the total carotene content of the above selections. Other carotenes present are β -, δ -, and γ -carotene and lycopene.

Tetrahydrophytoene

Relatively few analyses have been made for this compound, and thus far no attempt has been made to develop tomato selections of a high tetrahydrophytoene content. It is known, however, that this compound is present in varying amounts in a number of different tomato selections. One selection in particular, P. I. 91458, a pale yellow-fruited tomato, has been analyzed several times for its tetrahydrophytoene content. Fruit of this selection contained between 25–50 $\mu\text{g.}$ of this

TABLE I
The Maximum Content of Various Carotenes and Colorless Polyenes Found in Tomato Selections Bred for a High Content of These Compounds

| | $\mu\text{g./g.}$ | Vitamin-A active |
|-----------------------|-------------------|------------------|
| Lycopene | 300–400 | no |
| β -Carotene | 120 | yes |
| Tetrahydrolycopene | — | " |
| γ -Carotene | 11 | yes |
| δ -Carotene | 40 | no |
| Prolycopene | 26 | no |
| Protetrahydrolycopene | 16 | no |
| ζ -Carotene | 85 | no |
| α -Carotene | 9 | yes |
| Phytofluene | 30 | no |
| Phytoene | 43 | " |
| Tetrahydrophytoene | 25–50 | " |

" These compounds have not been bioassayed. There is little chance, however, on the basis of their structures that they have vitamin A activity.

compound, and in addition they contained less than 5 μ g. of other colorless polyenes and carotenes, most of which was β -carotene.

It is now suspected that tetrahydrophytoene⁹ is eicosahydrolycopene. It is also suspected that selection P. I. 91458 will prove to be homozygous for the major factors for the synthesis of tetrahydrophytoene.

Table I gives the maximum content of the various carotenes and colorless polyenes reported in this paper and indicates whether these compounds have vitamin A activity. It will be noted that the selections containing a high content of β -carotene are the only ones of practical nutritional significance. The selections containing the other colorless polyenes and carotenes are of significance as source material for the isolation of specific compounds or as genetic material for inheritance studies.

DISCUSSION

The development of selections of a relatively high content of certain carotenes and colorless polyenes has been invaluable to studies on the structure of these compounds. This has been especially true in the case of compounds such as ζ -carotene (20), δ -carotene (11), and phytoene (9), for these compounds are found in only very small amounts in other sources.

Three major genes are known to be involved in the synthesis of tomato carotenes. These genes are *R*, *T*, and *B* (4,18,21). All of these genes are necessary for the formation of β -carotene. Genes *R* and *T* are necessary for the formation of lycopene whereas *R* is necessary for the synthesis of the pigments of the Tangerine tomato. The absence of *R* results in tomatoes of a very low carotene content.

Selections homozygous for β -carotene or lycopene exist. In addition, the selection P. I. 91458 is homozygous for a low total carotene content, which quite possibly may eventually be found to involve the genetic inability of the selection to go beyond the eicosahydrolycopene stage, to any appreciable extent, in carotene synthesis. One other homozygous, pigment-type of tomato is known. This type is represented by the Tangerine and Golden Jubilee varieties. This type differs genetically from the lycopene type by a single recessive major gene, and it differs chemically by the presence in these fruits of prolycopene, protetrahydrolycopene, tetrahydrolycopene, ζ -carotene, phytofluene, and phytoene. The present work represents, in part, an attempt to separate the genetic

⁹ The structure of this compound is under investigation by W. J. Rabourn and J. W. Porter.

factors for the pigments and colorless polyenes of the Tangerine type. As yet new homozygous color types have not been obtained, but progress has been made. Most noteworthy is the high content of ζ -carotene in some of the experimental selections.

Other carotenes, besides those in the Tangerine group, lycopene, and β -carotene, are α -, δ -, and γ -carotene. It is extremely doubtful that selections containing only γ - or α -carotene will be developed. There seems to be a good chance, however, that true breeding selections containing mainly δ -carotene will be developed. If so, such a selection as in the case of the β -carotene-type tomatoes, will represent a new, homozygous, pigment-type of tomato.

Attempts to increase the tetrahydrolycopene¹⁰ content of tomato selections by breeding have not been made. Neither have attempts been made to increase the content of *cis* isomers, other than prolycopene and protetrahydrolycopene, in tomatoes.

The loss of the "Unidentified II" pigment of Porter and Zscheile (1) in advanced generations of the selection containing this compound is reported. It should be stated at this time that the pigment, "Unidentified II," contained eight conjugated double bonds. To the authors' knowledge this is the shortest even number of double bonds reported in the carotene series to date.

It should be noted that a few of the selections reported in this paper are not as high in content of a particular carotene as those reported in an earlier publication. Apparently the shifting of factors that occurs in inbreeding has resulted in some cases, in the relatively small populations which have been examined, in genetic combinations not quite as favorable for the production of a particular pigment as the genetic combination in the parent.

The development of selections containing a high content of one or more of the colorless polyenes and carotenes may prove to be an example of a general method for studying the mechanism of the biosynthetic processes in plants. This method of studying biosynthesis, in which selections from the crosses of genetically diverse stocks are made, is based upon the assumption that the parent stocks will differ at more than two loci which affect the biosynthetic process in question. The compounds in these selections will then represent various stages in the synthesis of the compound which is the end product of the synthetic

¹⁰ This compound is considered to be the same as the pigment A of Bonner *et al.* (22) and Neurosporene of Haxo (23).

process in question. If chemical or physical methods can be devised for detecting these compounds, their position in the biochemical scheme can then be ascertained by genetic means, and, possibly, ultimately proven by enzymatic studies.

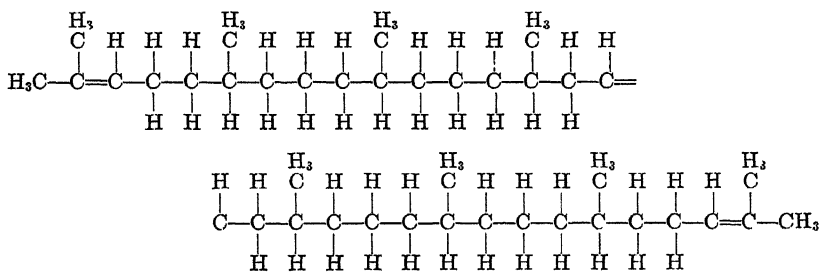
It is suggested that the proposed method may prove to be more satisfactory in the study of certain biochemical processes (pigment synthesis for example) than the method whereby mutations are produced through experimental means. In the case of artificially produced mutations the probability of producing a specifically desired mutation is very remote.

II. THE MECHANISM OF CAROTENOID BIOSYNTHESIS

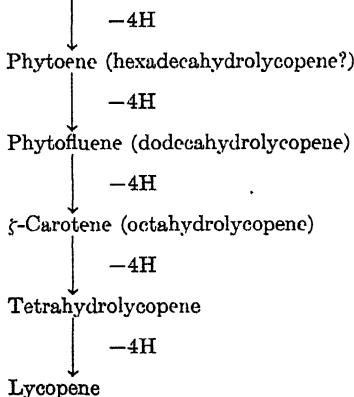
Considerable information has been gathered on the relationship of colorless polyenes and carotenes to one another. This information includes data on the solubility characteristics, adsorptive behavior, qualitative (number of conjugated double bonds) and quantitative light-absorption curves, C and H content, molecular weight, C-methyl groups, isopropylidene groups (including the identification of acetone), hydrogen absorption, and the behavior towards iodine in isomerization studies on all not previously well-characterized compounds with the exception of tetrahydrophytoene. Solubility characteristics, adsorptive behavior, and a qualitative light-absorption curve have been obtained for the latter compound. Other data include the optical rotations of phytofluene, δ - and ζ -carotenes and the bioassays noted in Table I. The inheritance relationships of the genes responsible for the four color types of tomatoes, the results of attempts to further breakdown the four genetic color types of tomatoes, and the results of hundreds of analyses of a wide variety of tomatoes for carotenes and colorless polyenes are also available. When this information is carefully considered, certain deductions can be made. These support and extend previous suggestions and evidence (2,19,22,24) that the carotenes are formed from colorless polyenes.

Some insight into the mechanism of carotene biosynthesis can be gained from an examination of the data obtained on the structural formulas of the carotenes and colorless polyenes. Present data indicate that phytofluene, ζ -carotene, tetrahydrolycopene, and lycopene form a series which differ from one another only in the number of conjugated double bonds in the molecule (6,17,20). This suggests they may be formed from one another by either reduction or dehydrogenation. Evidence on this point is supplied by inheritance studies involving crosses

of commercial varieties of high lycopene content to selections of the Tangerine type or to selections of the "low carotene," tetrahydrophytoene type. In each of these crosses the factors for red color (lycopene) are dominant. Pigments and colorless polyenes of the Tangerine type are not present or present only in traces in the hybrids of the first cross. The lycopene content in the hybrids of the second cross is not diminished from the content of this compound in the red parent. Each of these crosses segregates in the F_2 into three red and one orange, or one yellow, respectively. The fact that the factors for lycopene formation are dominant in both of these crosses strongly suggests that hydrogenation of lycopene to form the compounds of the Tangerine type or tetrahydrophytoene does not occur. Instead the dominance of the factors for lycopene implies lycopene is formed from these compounds by dehydrogenation. The following scheme is then postulated for lycopene formation. In this scheme an assumption is made that two C_{20} fragments combine to form a C_{40} compound.



Tetrahydrophytoene (eicosahydrolycopene?)

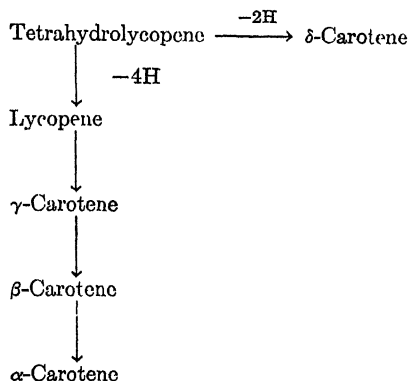


Since it is now generally accepted that a single gene controls the formation of an enzyme which in turn catalyzes a single reaction or type of reaction, one or more genes are involved in the dehydrogenation of tetrahydrophytoene to lycopene. The development of selections high in content of one or more of phytoene, phytofluene, ζ -carotene, and lycopene is compatible with the several-gene-hypothesis. The hypothesis that a single gene controls this series of reactions is consistent with the fact that each postulated reaction involves a dehydrogenation which could be carried on by a single enzyme. In this case the predominant carotene or colorless polyene of a tomato selection might then be determined by the nature and content of hydrogen acceptors present and not by a gene for a specific dehydrogenation (*i.e.*, phytofluene \rightarrow ζ -carotene).

A third alternative, for which some evidence is available, would suggest that the two major genes known to be necessary for lycopene formation function in the formation of two enzymes, one of which would bring about α - β dehydrogenation and the other, γ - δ dehydrogenation. Then tomatoes containing gene *R* but not gene *T* would have the series of pigments found in the Tangerine tomato. In tomatoes containing genes *R* and *T* the enzyme resulting from the presence of gene *T* would act upon phytoene to form a compound with four additional isolated double bonds¹¹ which would then be acted upon by the enzyme resulting from the presence of gene *R* with the resultant formation of lycopene.¹

Several carotenes do not have the open chain structure of lycopene (α -, β -, δ -, and γ -carotenes). Inheritance studies show that a single gene difference exists between selections which have principally lycopene and those which have almost 100% of β -carotene (4). Dominance of either of the pigments is not exhibited in the *F*₁ however, but since evidence indicates the formation of lycopene from more highly saturated compounds, it seems probable that β -carotene is formed from lycopene. Since γ -carotene is intermediate in structure to lycopene and β -carotene, it is assumed to be formed as an intermediate in the above reaction. A small conversion of β -carotene to α -carotene is assumed to occur in some selections. By virtue of its structure, δ -carotene is assumed to be formed from tetrahydrolycopene by dehydrogenation ($-2H$) and ring closure (at one end of the molecule). The following scheme shows the proposed relationship of the above compounds.

¹¹ Present evidence indicates this compound probably exists in red tomatoes (9).



Lycoxanthin and lycophyll also occur in tomatoes. These compounds are assumed to arise from lycopene as follows:

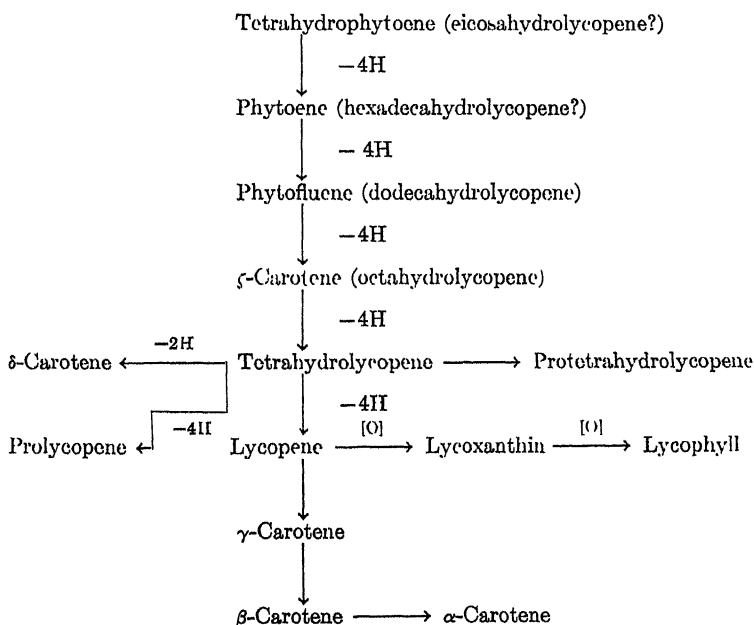
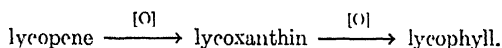


FIG. 1. Postulated pathway of carotenoid biosynthesis.

Since several of the compounds shown in Fig. 1 have been found in carrots and other yellow, orange, and red fruits and vegetables, it seems highly probable the same mechanism of carotene biosynthesis should be postulated for these fruits and vegetables. The absence of phytoene, phytofluene, ζ -carotene, and lycopene from green leaves indicates that a different mechanism for the biosynthesis of carotenes exists in chloroplasts. This may not be true, however, for tetrahydrophytoene has been found in green leaves (7).

SUMMARY

Lycopersicon selections have been developed, by crossing *Lycopersicon* species and then inbreeding and selecting, which contain the following amount of carotenes and colorless polyenes.

| | $\mu g / g$ | | $\mu g / g$ |
|-----------------------|-------------|--------------------|-------------|
| Lycopene | 300-400 | ζ -Carotene | 85 |
| β -Carotene | 120 | Phytofluene | 30 |
| γ -Carotene | 11 | Phytoene | 43 |
| δ -Carotene | 40 | α -Carotene | 9 |
| Prolycopene | 26 | Tetrahydrophytoene | 25-50 |
| Protetrahydrolycopene | 16 | | |

It is suggested that the method used in the development of the above selections may be of general value in the elucidation of biosynthetic mechanisms. The superiority of this method over the mutation method in the study of certain biosynthetic mechanisms is also suggested.

A scheme for the biosynthesis of carotenes in tomatoes is presented (Fig. 1). The order of the compounds in the biosynthetic scheme has been established on the basis of chemical structure. Evidence for the direction of the reactions has been supplied by studies on the inheritance of key compounds in the scheme. Three types of reactions are involved in the postulated scheme: dehydrogenation, ring formation, and oxidation.

It is suggested that the postulated scheme is probably valid for all yellow and red, carotene-containing, fruits and vegetables. The scheme may or may not be valid for green leaves. The detection of tetrahydrophytoene in green leaves may possibly validate the scheme.

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An Oxidative, Cyanide-Insensitive Enzyme System in the Chloroplasts of a Higher Plant

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INTRODUCTION

An oxidative enzyme system utilizing molecular oxygen has been found to be associated with the chloroplasts isolated from spinach leaves. The nature of the substrate which is oxidized has not yet been determined, but a notable feature of the system is that oxygen consumption is not inhibited by cyanide. Since the respiration of certain higher plant tissues has been reported to be cyanide-insensitive (4,5), the cyanide-stable oxidative system described below may be of importance.

EXPERIMENTAL AND RESULTS

Spinach leaves were rapidly frozen with solid carbon dioxide, and powdered in a mortar while still frozen. Manometric studies, carried out with the leaf powder suspended in phosphate or phthalate buffer, show a gas exchange with a characteristically high respiratory quotient (R. Q.) of 1.4 or greater. Suspensions of fresh tissue, homogenized in an Elvehjem type homogenizer until free of whole cells, exhibit the same type of activity. The oxygen uptake of tissue suspensions prepared in either way may equal 50–60% of that of intact spinach leaves. The observed gas exchange is unaffected by light, and is uninhibited by cyanide in concentrations up to 0.01 *M*. The system responsible for the observed activity is clearly not polyphenoloxidase, since the oxidation of dihydroxyphenylalanine by the latter enzyme is readily suppressed by 0.01 *M* cyanide (Table I). Furthermore, the cyanide-insensitive gas exchange of frozen or homogenized spinach leaves increases as the pH

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is lowered, and is maximal in the range pH 4.5–5.0. Polyphenoloxidase of spinach leaves, on the other hand, is much less active at pH 5.0 than at pH 6.8, and is almost completely inactive at pH 4.5. Both the pH range for optimum activity, and the localization of the enzyme within the chloroplasts (see below), distinguish the system here described from the cyanide-insensitive α -hydroxy acid oxidase reported by Clagett *et al.* (2).

Spinach leaves, whether freshly harvested or purchased in the market, show great variability in activity throughout the year. It has been discovered that the conditions under which the plants are grown determine whether or not the leaves will yield an active preparation. For example, when two comparable batches of spinach plants (which had been grown for 4 weeks under ordinary greenhouse conditions) were

TABLE I

The Effect of 0.01 M Cyanide on the Oxygen Uptake of Frozen Spinach-Leaf Suspensions in the Presence and Absence of 3,4-Dihydroxy-L-phenylalanine (Dopa)

Each manometer vessel contains: 800 mg frozen leaf powder; 1.0 ml. 0.1 M phthalate buffer, pH 4.5, or 1.0 ml. phthalate buffer containing 1 mg. dopa. Final pH, 5.0. Center well contains 0.2 ml. 2.0 M KCN in experiments with cyanide, and 0.2 ml. 10% KOH in all other experiments. Temp. 30°C.

| | Oxygen consumed in 10 min. cu.mm. | Oxygen uptake/ control per cent |
|--------------------|---|---------------------------------------|
| Control | 39.4 | 100 |
| KCN added | 36.0 | 91 |
| Dopa added | 58.0 | 147 |
| Dopa and KCN added | 37.8 | 96 |

held for 2 weeks in greenhouses maintained at 18°C. or 26°C., respectively, preparations from the former were found to be inactive, whereas preparations from plants held at the higher temperature were active. The minimum period required for such a temperature effect appears to be shorter than here reported. Variations in day length and light intensity are apparently not critical.

A heat-labile component of the system is entirely sedimented by centrifugation for 15 min. at 20,000 $\times g$. When sedimentation is incomplete, following centrifugation at lower speeds, neither the precipitate nor the supernatant alone shows high activity. Recombination restores full activity (Table IIa). The supernatant from rapidly centrifuged suspensions (20,000 $\times g$) is completely inactive. Differential centrifugation studies were carried out in which the activity of the precipitate

TABLE II

The Effect of Heat-Treated Supernatant on the Oxygen Uptake of Leaf-Homogenate Precipitates and of Whole Chloroplasts, Respectively

a. Eight g. frozen, powdered, leaves mixed with 7.0 ml. ice-cold distilled water and homogenized. Homogenate passed through sharkskin-grade filter paper in a small basket centrifuge. Final filtrate volume, with washings, 13.0 ml. Four-ml. aliquots centrifuged for 30 min. at $5200 \times g$. Supernatants decanted. Precipitates resuspended in distilled water or in supernatant, to final volume of 4.0 ml. Chlorophyll concentration determined as described by Granick (3) at wavelength of $667.5 \text{ m}\mu$, in Coleman Junior Spectrophotometer. Each manometer vessel contains: 2.0 ml. specified preparation; 1.0 ml. distilled water; 0.5 ml. 0.25 *M* phthalate buffer, pH 4.5. Final pH, 5.0; 0.2 ml. 10% KOH in center well. Temp. 30°C .

| | Oxygen consumed in 10 min. | Relative chlorophyll concentration | Activity/ unit chlorophyll | Activity/ total initial activity |
|--|----------------------------------|--|----------------------------------|--|
| | <i>cu. mm.</i> | | <i>per cent</i> | <i>per cent</i> |
| Filtered homogenate | 24.3 | 0.99 | 100.0 | 100.0 |
| Precipitate | 6.9 | 0.89 | 32.0 | 28.4 |
| Heated precipitate | 0.0 | 0.89 | 0.0 | 0.0 |
| Supernatant | 5.4 | 0.24 | 93.0 | 22.2 |
| Heated supernatant | 0.0 | 0.24 | 0.0 | 0.0 |
| Precipitate + supernatant | 23.2 | 1.00 | 95.0 | 96.0 |
| Precipitate + heated supernatant | 20.8 | 0.90 | 95.0 | 86.0 |
| Heated precipitate + heated supernatant | 0.0 | 0.90 | 0.0 | 0.0 |
| Filtered blend | 43.3 | 0.84 | 100.0 | — |
| Whole chloroplasts | 4.4 | 1.00 | 8.0 | — |
| Whole chloroplasts + heated supernatant | 34.8 | 1.00 | 68.0 | — |

TABLE II (Continued)

b. Twenty-four g. fresh spinach leaves ground in mortar with 35 ml. ice-cold 0.5 *M* sucrose. Ground material blended for 1 min. in cooled micro-Waring Blendor. Blend filtered through sharkskin-grade filter paper in basket centrifuge. Final filtrate volume 50.0 ml. Aliquot taken directly as "filtered blend." Forty ml. remaining filtrate centrifuged at $800 \times g$ for 30 min. Supernatant discarded. Precipitate twice resuspended in 50 ml. 0.5 *M* sucrose, and centrifuged. Final precipitate brought to 8.0 ml. with 0.5 *M* sucrose (whole chloroplasts). Each manometer vessel contains: 2.0 ml. filtered blend or whole chloroplast suspension; 1.0 ml. distilled water or 1.0 ml. twice-concentrated, boiled, and clarified supernatant; 0.5 ml. 0.25 *M* phthalate buffer, pH 4.5. Final pH, 5.0; 0.2 ml. 10% KOH in center well. Temp. 30°C .

was measured after suspending the latter in boiled and filtered supernatant, separately prepared. A close correlation is observed between the fraction of the total initial activity recovered in the precipitate, and the fraction of the total, initial chlorophyll content recovered in the same precipitate (Table III). In addition, whole chloroplasts, prepared as described by Granick (3), show little or no activity alone, but appreciable activity in the presence of the supernatant (Table IIb). This activity is enhanced when chloroplasts are homogenized following their preparation.

TABLE III

Distribution of Chloroplast Fragments and Enzymatic Activity Following Centrifugation

Filtered homogenate prepared as described in Table IIa. Four-ml. aliquots centrifuged for 15 min. at specified relative centrifugal force. Supernatants decanted. Precipitate brought to 4.0 ml. with separately-prepared, boiled, clarified supernatant. Aliquot used directly for chlorophyll determination. Each manometer vessel contains: 2.0 ml. suspension; 1.0 ml. distilled water; 0.5 ml. 0.25 *M* phthalate buffer, pH 4.5. Final pH, 5.0; 0.2 ml. 10% KOH in center well. Temp. 30°C.

| Fraction precipitated at given centrifugal force | Per cent of original chlorophyll content | Oxygen consumed in 40 min. | Activity |
|--|--|----------------------------|-----------------|
| $\times g$ | | <i>cu. mm.</i> | <i>per cent</i> |
| 0(original) | 100 | 181 | 100 |
| 20,000 | 100 | 179 | 99 |
| 6000 | 66 | 112 | 62 |
| 2100 | 53 | 101 | 56 |
| 1100 | 28 | 55 | 30 |

The oxygen consumption of chloroplasts and chloroplast fragments, sedimented by high speed centrifugation and resuspended in phthalate buffer, remains unaffected by additions of glucose, malate, pyruvate, or succinate, whether in the presence or absence of added adenosine triphosphate (ATP), Coenzyme I, and Mg^{++} . Furthermore, certain respiratory inhibitors, at concentrations which drastically affect respiration *in vivo* (1) have little or no effect upon the oxygen uptake of the above mentioned preparations. Whereas the per cent inhibition of oxygen consumption *in vivo* by malonic acid (5 mg./ml.), iodoacetic acid (0.1 mg./ml.), and NaF (0.1 mg./ml.), is 93%, 62%, and 75%, respectively, the oxygen consumption of chloroplast preparations is correspondingly inhibited 0.0%, 14%, and 0.0%. Higher concentrations of iodoacetic

acid (1.0 mg./ml.) bring about inhibitions up to 80%, while NaF at a concentration of 1.0 mg./ml. brings about an inhibition of 32%.

DISCUSSION

Respiratory studies with tissues from a variety of higher plants have indicated the respiration to be cyanide-sensitive. However, several instances have been reported in which the respiration remains unaffected by cyanide. Of particular interest are those experiments with carrot leaves, in which immature leaves exhibit cyanide-sensitive respiration whereas mature leaves do not (4). It is noteworthy that the respiration of even the immature leaves is not completely inhibited by cyanide. Similarly, in experiments reported by Bonner and Wildman (1) and repeated by the author, spinach leaf respiration is inhibited but 75% by 0.01 *M* cyanide.

The high rate of oxygen utilization by tissue preparations herein described is most readily observed when the pH is adjusted to approximately 5.0. If the pH is kept at 6.6, the pH of the untreated brei, the oxygen utilization is but 20–30% that of the intact tissue. Since this oxygen consumption is cyanide-insensitive, the possibility suggests itself that the oxygen uptake which persists after freezing or homogenization represents the fraction of the respiration which, *in vivo*, is unaffected by cyanide. Alternatively, the enzyme system described above may normally be an intermediate in a respiratory chain, and become autoxidizable only after the continuity of this chain is destroyed by manipulation of the tissue. The information presently available does not distinguish between these alternatives.

SUMMARY

Spinach leaves rapidly frozen with solid carbon dioxide, or homogenized directly to the cell-free state, were shown to exhibit a cyanide-insensitive gas exchange with an R. Q. of 1.4 or greater. A heat-labile component of the system responsible for this gas exchange was readily sedimented by centrifugation and was shown to be contained in the chloroplasts, or chloroplast fragments. A heat-stable component was found to remain in the supernatant following centrifugation. The nature of the latter substance has not yet been elucidated. Active preparations were obtainable from the leaves of spinach plants grown at 26°C., but not from leaves of plants grown at 18°C.

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Bradykinin, Assay of Purification¹

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INTRODUCTION

In previous reports from this laboratory (1,2,3) it has been shown that plasma globulins release a hypotensive and smooth muscle-stimulating principle, "bradykinin," when incubated with the venom of *Bothrops jararaca* or with crystalline trypsin. Since further contact with the venom or with trypsin destroys its activity, the material appeared as a polypeptide. The chromatographic analysis on filter paper (4), performed in our laboratory by Dr. Sylvia O. Andrade (5), showed the presence of at least 12 amino acid residues: lysine, histidine, arginine, aspartic acid, serine, glycine, threonine, alanine, proline, leucine, and phenylalanine. Tyrosine is absent, and colorimetric testing on the most highly purified preparations showed the presence of tryptophan. Bradykinin is slowly dialyzable through cellophane paper, resistant to prolonged boiling in neutral or slightly acid solution, but rapidly destroyed by boiling in weak alkaline solution. It can, therefore, be distinguished from the thermolabile kallikrein (6) and the active principle isolated from platelets—SMC or thrombocytin (7,8)—that appear to be more resistant to boiling in alkaline than in acid solution.

In the present paper, attempts to further purify "crude" bradykinin will be described.

MATERIALS AND METHODS

Units and Bioassay

For comparison, we have decided to define one unit of bradykinin as the activity contained in 1 mg. of the *Pool I* preparation,⁴ the first homogenized "crude" brady-

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⁴ The activity of *Pool I* is about twice that of *Brady I*, described in a previous publication (2).

kinin preparation. We have avoided defining the unit in terms of biological activity, since the best biological assay for testing bradykinin activity is the guinea pig gut suspended in a Tyrode bath. The sensitivity of this preparation varies widely from one piece to the other and also according to the capacity of the chamber containing the biological preparation. In this laboratory, the preparation is set up uniformly by taking a piece of ileum of 2.5–3.0 cm., from a guinea pig of 230–300 g. body weight. The piece of ileum is carefully washed with warm Tyrode solution and fixed in a chamber of 7 cc. capacity. The bath is kept at 36–37°C. The frontal writing lever used was designed according to Schild's specifications (9), with an overcharge of 0.8–1.0 g.

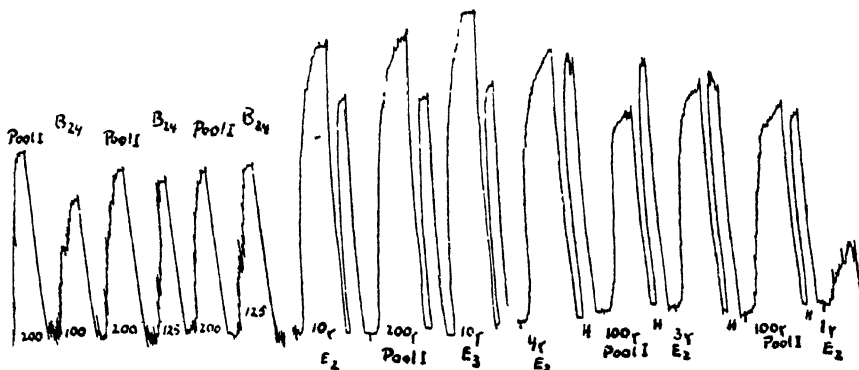


FIG. 1. Biological assay of bradykinin upon the guinea pig gut. In the first panel, 125 μ g. of a crude bradykinin preparation (B_{24}) was matched against 100 μ g. of Pool I (one unit/mg.); in the middle panel, two eluates (E_2 and E_3) with an activity around 20 times the Pool I were assayed upon the gut; in the third panel, another eluate E_2 assaying 33 units/mg. was added to the bath in three different doses (4 μ g.; 3 μ g.; and 1 μ g.). The muscle distinctly reacted to 1 μ g. (final dilution 0.14 μ g./ml.). $H = 0.30$ cc. of histamine base 0.5 p.p.m.

Under such conditions the useful range of dosage for a bradykinin assay lay between 200 μ g. and 500 μ g. of Pool I, or 0.2–0.5 units of bradykinin. It was seldom necessary to shift the dosage to higher levels in order to remain within the limits of the useful range of the gut. Figure 1 gives an example of a potency determination of three different preparations of bradykinin. In most cases, the bracketing of doses of the unknown with doses of the standard was employed to follow the assays of purification. Later, a four-point assay was developed, mainly along the lines of the assay described by Schild (10) for histamine estimation. The log ratio of potency, its standard error and confidence limits are calculated from the heights in millimeters of the responses, using factorial coefficients (11).

Chromatographic Experiments

Permutit or aluminum oxide, according to Brockmann, were employed in columns with variable length and diameter, according to the amount of material to be purified.

For the experiment described in Table III, a column of aluminum oxide made up with 24 g. of adsorbent in a glass column of 24 mm. diameter and 75 mm. length was employed. The Brockmann's aluminum oxide (Merck and Co., Rahway, N. J.) was previously washed with dilute acetic acid, followed by water, and dried. Before starting chromatography, the column was washed with the solvent (ethyl alcohol, 90 or 70%). The best results were obtained by using as starting material (original) the preparation obtained through acetic acid extraction and ethyl ether precipitation, as will be described in the following.

RESULTS

Acetic Acid Extraction

Bradykinin is readily soluble in glacial acetic acid and can be quantitatively precipitated by addition of a great excess of ethyl ether. The crude preparation is extracted several times with an appropriate volume of glacial acetic acid and the supernatants collected in the centrifuge. Upon addition of eight volumes of ethyl ether, a heavy precipitate is formed that is collected by centrifugation. The supernatant is discarded and the precipitate extracted twice with smaller volumes of glacial acetic acid and reprecipitated with ethyl ether. A typical experiment is

TABLE I

Assay of Purification of Bradykinin by Extraction With Glacial Acetic Acid and 80% Ethyl Alcohol

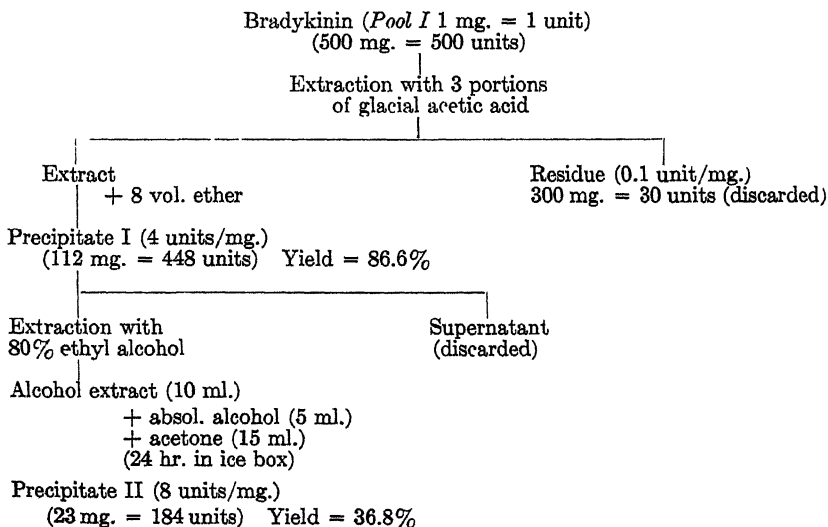


TABLE II

Assay of Purification of Bradykinin by Using Organic Solvents

| Assay of purification | Weight | Activity | Total activity | Yield |
|---|------------|------------------|----------------|----------|
| | <i>mg.</i> | <i>units/mg.</i> | <i>units</i> | <i>%</i> |
| Crude bradykinin (original) | 460 | 3 | 1380 | |
| Glacial acetic acid + ethyl ether: | | | | |
| 1st extraction: | 184 | 7 | 1288 | 93 |
| 2nd extraction: | 130 | 10 | 1300 | 94 |
| 3rd extraction: | 103 | 10 | 1030 | 74 |
| Extraction with 80% ethyl alcohol and pptn. with acetone: (two extractions) | 40 | 15 | 600 | 43 |

presented in Table I. As can be seen, activity is easily raised up to four times the "crude" preparation following extraction with glacial acetic acid. As indicated in the table, a further extraction with alcohol (80%) and precipitation with an alcohol-acetone mixture, although increasing the activity from 4 units/mg. to 8 units/mg. was accompanied by a considerable loss of material. Table II shows another assay of the kind in which 460 mg. of crude bradykinin (3 units/mg.) was submitted to three successive purifications with glacial acetic acid extraction and ethyl ether precipitation. After the second extraction with glacial acetic acid, activity increased to 10 units/mg., and 94% of the original material was recovered. A third extraction with acetic acid did not further increase the activity, but the yield dropped to 74% of the original. Two extractions with alcohol and precipitation with acetone raised the activity to 14-15 units/mg., at the cost of a heavy loss of material.

Solubility of Bradykinin in Organic Solvents

Bradykinin is almost insoluble in most organic solvents assayed: acetone, chloroform, ethyl ether, ethyl methyl ketone, petroleum ether, butyl alcohol, amyl alcohol, and ethyl acetate; it is soluble in glacial acetic acid, in a 10% solution of trichloroacetic acid, 70% ethyl alcohol, and in hot methyl alcohol; it is less soluble in 90% ethyl alcohol or cold methyl alcohol. The diagram of Fig. 2 shows the relative solubilities of bradykinin in different organic solvents.

Assays of Purification by Chromatography

Bradykinin is rapidly removed from a water solution on passage over charcoal or permutit. Partial elution from charcoal can be done by treatment with glacial acetic acid. The yields were, however, exceedingly small. From permutit, adsorbed bradykinin could be eluted by treatment with a concentrated (up to 20%) solution of NaCl. The difficulty of a complete removal of the salt considerably limited the usefulness of this method of purification.

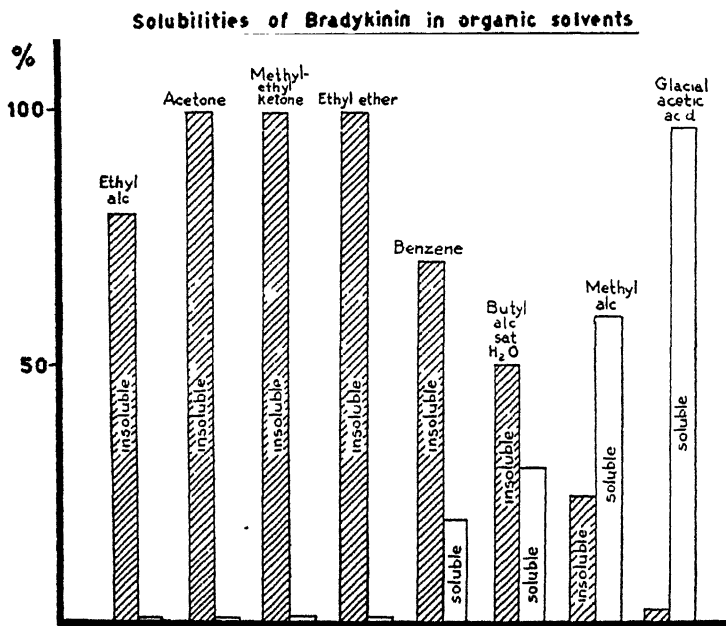


FIG. 2. Solubilities of bradykinin in different organic solvents at room temperature.

Bradykinin is completely removed from a 90% ethyl alcohol solution on a first passage through the column of aluminum oxide. Very poor yields were obtained through elution with lower alcohol concentrations. Better results were obtained by avoiding retention of the active material in the column. In consequence, we have adopted as a routine procedure the passing of a solution of bradykinin in 70% alcohol as rapidly as possible through the column of aluminum oxide. The latter retains

most of the pigment and probably also some of the peptides that contaminate bradykinin. The solution passing through the column was concentrated *in vacuo* in small flasks, weighed, and the activity estimated. Table III shows the results of a chromatographic experiment in which 336 mg. of bradykinin (5 units/mg.) were passed through a column of 24 g. of aluminum oxide.

TABLE III

Chromatographic Analysis of a Bradykinin Preparation in Aluminum Oxide, Using as Original Solvent 70% Ethyl Alcohol

| Fraction | Solvent | Vol. of solvent | Weight of dried material | Activity | Total activity | Yield |
|-----------------------|-------------|-----------------|--------------------------|-------------|----------------|----------------------|
| Original | Alcohol 70% | ml. 40 | mg. 236 | units/mg. 5 | units 1180 | |
| Eluates: | | | | | | |
| <i>E</i> ₁ | Alcohol 70% | 40 | 10.6 | 20 | 212 | 791 units (67.0%) |
| <i>E</i> ₂ | Alcohol 70% | 40 | 13.3 | 30 | 399 | |
| <i>E</i> ₃ | Alcohol 70% | 40 | 9.0 | 20 | 180 | |
| <i>E</i> ₄ | Alcohol 50% | 50 | 7.9 | 15 | 118 | 158 units |
| <i>E</i> ₅ | Water | 60 | 20.0 | 2 | 40 | (13.4%) |
| | | | | Totals | 949 | 80.4% |

As shown in Table III, after the first passage, 67% of the previous total activity could be recovered with an increase of potency from 5 units/mg. to 20–30 units/mg. After washing the column with a lower alcohol concentration, 118 units more could be recovered, and 40 units more by further washing with distilled water. All fractions totaled 80.4% of the activity contained in the original solution.

DISCUSSION

From the experimental evidence available at the moment, bradykinin appears to be constituted of several amino acids, forming a long chain that passes slowly through cellophane paper. It is soluble in 10% trichloroacetic acid, in glacial acetic acid, and in hot methyl alcohol. So far, the latter two are the only organic solvents in which bradykinin can be completely dissolved in the absence of moisture. It is easily retained by the common laboratory adsorbents (charcoal, aluminum

oxide, Lloyd's reagent, *etc.*). Its elution is difficult and is accompanied by a considerable loss of material. The pigment and part of the impurities are retained by the column of aluminum oxide when the original solution is made up in 70% ethyl alcohol.

The most active preparations so far obtained, assaying 60 units/mg., produced a perceptible response of the guinea pig gut when added to the bath in doses as small as 1 or 2 μ g. The final concentration of the material in the bath, would therefore be *ca.* 0.33 or 0.14 p.p.m. Since we have no indication that the material is pure (on the contrary, it still gives a faint but distinct indication of contamination when tested for ammonium sulfate), we are bound to assume that bradykinin is a powerful pharmacological agent. On the other hand, if one assumes that the molecular weight of bradykinin is above 5000 and below 10,000, a conclusion warranted by preliminary experiments of diffusion of bradykinin through sintered glass membranes (12) performed in our laboratory by Dr. C. R. Diniz, we have reasons to be impressed by the extraordinary activity of the pharmacodynamic grouping that is responsible for the activity of bradykinin. In fact, if its molecule is taken as 45 times as large as that of histamine, for instance, the activity of the best preparations obtained in our laboratory would exceed by a factor of 3 or 4, on a molar basis, the activity of histamine (the usual threshold sensitivity of our guinea pig preparations to histamine, is around 0.014 p.p.m. concentration of the base in the Tyrode bath).

Among the many known peptides, only a few have any interesting pharmacological action. Hypertensin (13) or angiotonin (14), a hypertensive principle liberated from plasma globulins by kidney extracts (renin), was shown by Edman (15) to be constituted of several amino acid residues. Chemically, hypertensin is closely related to bradykinin; they can be sharply distinguished through their opposing effects upon the arterial blood pressure. It is, however, very difficult to separate them through fractional solubilization in organic solvents. Simple amino acids, as histidine and arginine, have been shown to produce pharmacological effects (16), when injected intravenously into laboratory animals, but the effect depends upon the administration of huge amounts that far exceed those of bradykinin for the same pharmacological activity.

SUMMARY

Assays of purification by using different organic solvents and chromatographic methods have been described.

Bradykinin is soluble in glacial acetic acid and quantitatively precipitated from solution by the addition of eight volumes of ethyl ether. Following two or more extractions with glacial acetic acid, the activity can be raised up to ten times the original.

When bradykinin in 70% ethyl alcohol solution passes through an aluminum oxide column, activity can be raised up to 40 or 60 times that of the "crude" preparation (*Pool I*).

Bradykinin is soluble in methyl alcohol, in 70% ethyl alcohol, but less so in 90% ethyl alcohol or absolute ethyl alcohol; it is completely insoluble in ethyl ether, acetone, chloroform, ethyl methyl ketone, petroleum ether, butyl alcohol, amyl alcohol, and ethyl acetate. It is completely soluble in a 10% solution of trichloroacetic acid.

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Studies on the Cyclophorase System. X. The Requirement for Pyridine Nucleotide¹

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INTRODUCTION

In previous communications of this series, evidence has been presented that the cyclophorase complex of enzymes in the mitochondria catalyzes the complete oxidation of pyruvic acid, fatty acids, and certain amino acids in the absence of any added oxidation-reduction coenzymes (1-4). In view of the fact that the isolated enzymes which catalyze many of the individual steps in these over-all processes are known to require the addition of pyridine nucleotide for their activity, it is of some importance to establish beyond reasonable doubt that a real difference exists between mitochondrial and isolated oxidases with respect to this requirement.

Other investigators who have employed mitochondrial preparations have also reported that various oxidative steps in freshly-prepared enzyme suspensions proceed in the absence of added pyridine nucleotide [cf. Hunter and Hixon (5) and Potter *et al.* (6)].

The present communication provides evidence from manometric and Thunberg measurements that seven oxidases of the cyclophorase system function maximally in the absence of added pyridine nucleotide. The possibility that there is present initially an excess of free pyridine nucleotide is shown to be untenable. However, the presence of bound forms of the pyridine nucleotides is demonstrable and the stability of these bound forms under various experimental conditions has been studied.

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³ With the technical assistance of Irene Rechnitz and Muriel Feigelson.

EXPERIMENTAL

Pyridine Nucleotides

Diphosphopyridine nucleotide (DPN) of about 40% purity was obtained from the Schwarz Laboratories. The actual concentrations of our solutions were first determined by reducing the coenzyme with hydrosulfite, and then determining the concentration of reduced DPN spectrophotometrically at 340 $m\mu$ assuming a molar absorption coefficient of $6.22 \times 10^4 \text{ cm}^2 \cdot \text{mole}^{-1}$ (11). The details of the method are essentially those of Gutcho and Stewart (12). Triphosphopyridine nucleotide (TPN) was prepared by the method of LePage and Mueller (13).

Enzymatic Assay of DPN

When assaying nonturbid solutions for DPN the spectrophotometric procedure of Horecker and Kornberg (11) was used with slight modification. Essentially DPN becomes reduced by ethyl alcohol in the presence of a purified yeast alcohol dehydrogenase with semicarbazide to trap the acetaldehyde. Where turbid solutions had to be assayed, the malic apoöxidase test system was used routinely. The split malic enzyme was prepared from pig heart muscle by the method of Stumpf *et al.* (14). At the pH 5.4 precipitation stage, the precipitate was resuspended in dilute acetic acid buffer of pH 5.4 and then centrifuged down. This extra washing reduced the blanks to negligible values. Curve A of Fig. 1 shows the rate of reduction of 2,6-dichloro-

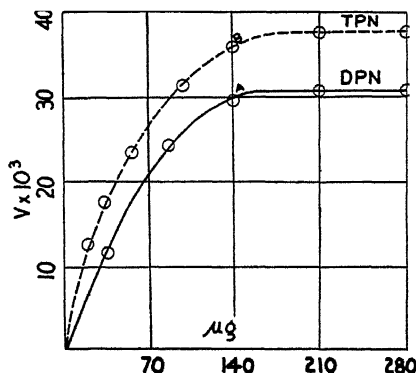


Fig. 1. Curve A: DPN test system—malic apoöxidase. Each Thunberg tube contained 0.3 ml. of the pig heart preparation (cf. experimental section), 2.0 ml. of 0.2 *M* 2-amino-2-methyl-1,3-propanediol (pH 9.5), 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol, 0.2 ml. of 1.0 *M* DL-malate, 0.1 ml. of capryl alcohol, DPN as indicated, and water to make 4.0 ml. Curve B: TPN test system—citric apoöxidase. Each Thunberg tube contained 1.0 ml. of the pig heart preparation (cf. experimental section), 2.0 ml. of 0.1 *M* phosphate buffer (pH 7.5), 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol, 0.03 ml. of 0.1 *M* manganous chloride, 1.0 ml. of 0.1 *M* citrate, 0.1 ml. of capryl alcohol, TPN as indicated, and water to make 5.5 ml.

phenolindophenol as a function of the concentration of DPN. Full experimental details are provided in the legend for the figure. Boiled extracts of liver or kidney cyclophorase contain substances which reduce the dye at pH 7. However, at pH 9.5 and in presence of capryl alcohol this blank reaction (in the absence of added malate) was completely eliminated. Assays should be made of DPN at concentrations which fall on the ascending part of the curve where sensitivity of the method is greatest. Under the conditions of the test, TPN showed about 10% of the activity of DPN in equivalent concentration.

Enzymatic Assay of TPN

With nonturbid solutions the TPN content was taken as the difference between the total pyridine nucleotide content, as determined spectrophotometrically after reduction with hydrosulfite, and the DPN content, as determined spectrophotometrically after reduction with alcohol in the presence of the alcohol dehydrogenase system. With nonturbid solutions and for rapid determination, TPN can be assayed in the isocitric apoöxidase system of pig heart muscle. The same pig heart preparation can be used as the source of both the malic and citric apoöxidase. Curve B of Fig. 2 shows the velocity of 2,6-dichlorophenolindophenol reduction as a function of the TPN concentration. It is to be noted that DPN in concentrations equivalent to TPN estimated in this test system to the extent of about 10%. While the citric apoöxidase test system has proved to be valuable for estimation of TPN in purified extracts it has a drawback when tested with boiled extracts of liver or kidney cyclophorase gel. These boiled extracts are able to reduce the indicator directly under the conditions of the test and no satisfactory way has been found to correct for this blank interference. For this reason the hexosemonophosphate apoöxidase system of yeast (15) has been used as an alternative test system for the assay of TPN in boiled extracts

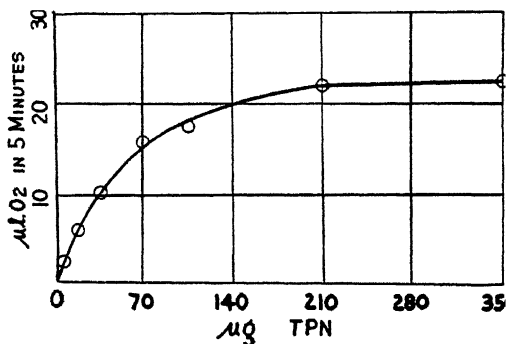


FIG. 2. TPN test system: glucose-6-phosphate apoöxidase. Each manometer cup contained 0.5 ml. of the yeast enzyme (1.5 ml. of dried cake of precipitate to 15 ml. of 1.0 *M* phosphate buffer of pH 7.2), 0.3 ml. of 0.1 *M* glucose-6-phosphate, 0.1 ml. of 20% alcohol, alkali in center well, and water to a final volume of 4.0 ml. Oxygen in gas space. Temp. 38°C.

of cyclophorase gel. This enzyme was prepared from yeast juice (1 part dried brewer's yeast (kindly supplied by the Pabst Brewery, Milwaukee) to 3 parts 0.1 *M* phosphate buffer of pH 7.2, incubated at 38° for 1 hr.). The enzyme was precipitated at 60% saturated ammonium sulfate and the precipitate was pressed dry with filter paper. The enzyme thus prepared contains the two components necessary for making aerobic measurements, viz., the apoxidase and the diaphorase. Fig. 2 shows the rate of oxygen uptake as a function of the TPN concentration. DPN does not estimate in the test system.

Anaerobic Thunberg Technique

2,6-Dichlorophenolindophenol has been recommended by Haas (16) for Thunberg studies. We have found this indicator eminently suited for this investigation. The conventional Thunberg technique was used in these studies. After evacuation of the tubes with a vacuum pump for 5 min., the tubes were immersed for 2 min. in a water bath at 38°C., and then the dye in the side arm was quickly mixed with the enzyme solution in the tube. The time for complete decolorization was then noted.

RESULTS

Requirement for Pyridine Nucleotide

When the cyclophorase system of rabbit kidney or liver has been freshly prepared it is found that the rate of oxidation (as measured in anaerobic Thunberg experiments) of lactate, malate, α -ketoglutarate, citrate, proline, glutamate, and β -hydroxybutyrate is not accelerated to any measurable degree by the addition of one or the other of the pyridine nucleotides (cf. Table I). In fact, each of the oxidases appears to be slightly inhibited by the pyridine nucleotide. In order to achieve the results of Table I several precautions had to be taken. The kidneys were ground in 0.01 *M* fluoride-0.9% potassium chloride to minimize autolytic processes. The enzyme gel was tested immediately after preparation. Finally the equilibration period prior to the introduction of the dye from the side arm of the Thunberg tube was eliminated. When any one of these precautions is omitted it is possible to demonstrate small but definitely positive effects of added pyridine nucleotide. The malic oxidase is one of the first to show a requirement for pyridine nucleotide. Table II considers the precautions necessary to insure that there is no response to the addition of pyridine nucleotide. In manometric experiments with several substrates extending over a period of more than 60 min., little or no effect of added pyridine nucleotide is demonstrable (cf. Table III).

In the course of comminuting either kidney or liver in a Waring Blendor there is a disruption of the mitochondrial units by the shearing

TABLE I

*Effect of DPN or TPN^a on Rate of Oxidation of Substrates
in the Kidney Cyclophorase System*

Each Thunberg tube contained 0.5 ml. of freshly prepared kidney enzyme at the third residue stage (R₃K),^b 0.3 ml. of 0.1 *M* phosphate buffer of pH 7.3, 0.4 ml. of 0.1 *M* substrate, and 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol in a total volume of 2.0 ml. The level of DPN or TPN^a was 150 μ g. of nucleotide per tube. The velocities for all experimental tubes have been corrected for the appropriate blanks without added substrate. The kidneys were mixed in the Waring Blendor in 0.9% potassium chloride which was 0.01 *M* with respect to sodium fluoride. Usual Thunberg technique, 38°C. Velocity is defined as the reciprocal of reduction time.

| Substrate | Velocity of dye reduction ($\text{sec.}^{-1} \times 10^3$) | | |
|------------------------------|--|-----------------------|-----------------------|
| | Without coenzyme | With DPN ^a | With TPN ^a |
| α -Ketoglutarate | 62.1 | | 58.6 |
| DL-Malate | 27.8 | 26.1 | |
| Citrate | 55.5 | | 37.3 |
| DL-Lactate | 29.4 | 24.6 | |
| DL- β -Hydroxybutyrate | 6.8 | 4.2 | |
| L-Proline | 16.1 | 13.0 | |
| L-Glutamate | 37.0 | 35.3 | |

^a DPN and TPN refer, respectively, to di- and triphosphopyridine nucleotide.

^b R₃K and R₃L indicate a thrice-washed cyclophorase residue of rabbit kidney and liver, respectively.

TABLE II

Effect of Pyridine Nucleotide on the Activity of Malic Oxidase

Each Thunberg tube contained 1.0 ml. of R₃K, 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.02 *M* magnesium chloride, 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.5) 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol (side arm), 500 μ g. of DPN where specified, 0.5 ml. of 0.1 *M* DL-malate, 1.0 ml. of 0.33 *M* fluoride (in Expts. 2 and 3), and water to make 5.5 ml.

A blank in each of the three experiments, consisting of DPN but no malate, gave a negligible contribution.

| Conditions | | Reaction velocity ($\text{sec.}^{-1} \times 10^3$) | |
|--------------------|----------------------|--|-------------|
| Incubation | Reaction temperature | With DPN | Without DPN |
| | °C. | | |
| 1. Two min. at 38° | 38 | 27.0 | 23.2 |
| 2. None | 38 | 21.7 | 20.4 |
| 3. None | 20 | 4.2 | 5.0 |

TABLE III
*Effect of DPN or TPN on Rate of Oxidation of Substrates
 of Cyclophorase System*

Each manometer vessel contained 1.0 ml. of kidney enzyme (R_2K), 0.3 ml. of 0.1 *M* substrate, 350 μ g. of DPN or TPN, 0.3 ml. of 0.1 *M* phosphate buffer of pH 7.5, 0.2 ml. of 0.02 *M* magnesium chloride, 0.2 ml. of 0.01 *M* adenosine-5-phosphate (adenylic acid), and 0.05 ml. of 0.33 *M* sodium fluoride. Alkali in center well; oxygen in gas space; 38°C. All values for oxygen uptake have been corrected for the appropriate blanks without added substrate.

| Time | Oxygen uptake (cu. mm.) | | | | | |
|------------|-------------------------|----------|------------------|----------|------------------|----------|
| | α -Ketoglutarate | | Malate | | Citrate | |
| | Without coenzyme | With DPN | Without coenzyme | With DPN | Without coenzyme | With TPN |
| <i>mn.</i> | | | | | | |
| 10 | 237 | 232 | 130 | 126 | 237 | 246 |
| 20 | 499 | 495 | 257 | 251 | 490 | 492 |
| 30 | 701 | 716 | 375 | 367 | 679 | 667 |
| 40 | 857 | 880 | 482 | 465 | 830 | 808 |
| 50 | 968 | 1032 | 583 | 555 | 948 | 947 |
| 60 | 1095 | 1216 | 660 | 616 | 1045 | 1086 |

action of the cutting blades (7). In consequence not all the particulate elements are sedimented in the gravitational field which is adequate to sediment the mitochondrial bodies with which the cyclophorase gel is associated. By increasing the gravitational field these smaller particles can be sedimented and washed. This fraction is usually referred to as the microsomal fraction,⁴ and the oxidases of the fraction prepared by this particular method show essentially no requirement for pyridine nucleotide (cf. Table IV).

Presence of DPN in Liver and Kidney Cyclophorase

The possibility is present, of course, that free pyridine nucleotide is not readily washed out of the cyclophorase gel owing to the highly

⁴ The fact that mitochondria can be fragmented into smaller particles, whose rate of sedimentation in a gravitational field corresponds with that of microsomes, does not necessarily imply that microsomes do not exist preformed in the cell. That may or may not be true. As long as microsomes can be characterized solely on the basis of their physical dimensions and rate of sedimentation, there is no way of distinguishing between microsomes which are formed by comminution and those which may be preformed.

viscous nature and occluding properties of the gel. In this section data will be presented which bears on this possibility.

In the washed kidney particles at the third residue stage (R_3K), the DPN content is roughly 140–170 $\mu\text{g./ml.}$, whereas at the third residue stage of liver (R_3L), the value is 105–140 $\mu\text{g./ml.}$ On a dry weight basis this would correspond approximately to 3.7 mg./g. for kidney cyclophorase and 1.6 mg./g. for liver cyclophorase. These estimations were carried out by heating an aliquot of either R_3K or R_3L for 1 min. at 100° and determining the DPN content of the entire coagulated suspen-

TABLE IV
*Effect of Pyridine Nucleotide on the Activity of Oxidases
in Microsomal Fraction*

Rabbit kidney was homogenized for 7 min. with 5 vol. of 0.9% KCl which was made 0.01 M with respect to sodium fluoride. The pH was maintained throughout between 7 and 8 by addition of alkali and the temperature was kept at 0° by frequent additions of frozen cubes of the salt solution. The homogenate was centrifuged at 0° for 10 min. at 4000 r.p.m., in the conical 8-place head of the International Equipment Company. The supernatant fluid was then centrifuged in the high speed attachment at 18,500 r.p.m. for 20 min. The sediment was resuspended in 0.9% potassium chloride and recentrifuged at the same speed. Details of the tests as in legend for Table I.

| Oxidase | Velocity ($\text{sec.}^{-1} \times 10^3$) | | |
|------------------------|---|----------|----------|
| | Without coenzyme | With DPN | With TPN |
| Lactic | 29.5 | 26.0 | 3.9 |
| Proline | 4.0 | 3.6 | |
| α -Ketoglutaric | 21.6 | 17.7 | |
| Citric | 3.5 | | |
| Glutamic | 4.8 | 4.4 | |

sion in the standard DPN-deficient malic oxidase test system (cf. experimental section) by comparison with known amounts of DPN. The endogenous DPN is firmly bound in the cyclophorase preparations and cannot be washed out or diminished by repeated suspension and centrifugation of the gel in 0.9% potassium chloride in the cold. When DPN is added to the cyclophorase gel at 0° and then the gel is thoroughly washed, the DPN content corresponds to the original amount of endogenous DPN, the added DPN being completely washed out in the process.

TABLE V

Destruction of DPN by Cyclophorase System

Each manometer vessel contained 1.0 ml. of R_3K , 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.02 *M* magnesium chloride, 0.1 ml. of 0.1 *M* phosphate buffer of pH 7.5, and water to make a final volume of 3.0 ml. The substrate was α -ketoglutarate of which 40 micro moles was added per cup. Alkali in center well; oxygen in gas space; 38°C.

At the conclusion of the experiment a 1-ml. aliquot was withdrawn from each cup and the DPN content determined. The DPN values have been calculated for the entire contents.

| Sample | Additions | Time of run | Oxygen uptake | Initial DPN | | | Recovered DPN |
|--------|-----------------|-------------|---------------|-------------|----------|----------|---------------|
| | | | | Endogenous | Added | Total | |
| | | min. | cu. mm. | μ g. | μ g. | μ g. | μ g. |
| 1 | Substrate | 15 | 482 | 175 | 0 | 175 | 155 |
| 2 | None | 15 | 2 | 175 | 0 | 175 | 110 |
| 3 | Substrate + DPN | 15 | 478 | 175 | 240 | 415 | 290 |
| 4 | DPN | 15 | 8 | 175 | 240 | 415 | 175 |
| 5 | Substrate | 45 | 1370 | 175 | 0 | 175 | 155 |
| 6 | None | 45 | 0 | 175 | 0 | 175 | 65 |
| 7 | Substrate + DPN | 45 | 1290 | 175 | 240 | 415 | 155 |
| 8 | DPN | 45 | 10 | 175 | 240 | 415 | 55 |

When a cyclophorase gel is allowed to carry on an active oxidation, it can be shown that there is essentially no loss of endogenous DPN during the course of the oxidation, whereas without substrate much of the endogenous DPN is destroyed (cf. Table V). External DPN under the same conditions is rapidly destroyed as illustrated by samples 3, 4, 7, and 8 of the same table. Although some residual DPN, over and above the endogenous, still exists in each sample at the end of 15 min., prolongation of the experiment to 45 min. results in an almost identical level of DPN in samples 6 and 8, indicating complete destruction of external DPN by enzymes in the cyclophorase preparation.

Barron *et al.* (8) have investigated the destruction of DPN in various tissues, while Kornberg and Lindberg (9) have shown that the destruction of DPN in kidney residue is largely referable to a pyrophosphatase action.

Presence of TPN in Cyclophorase

Both liver and kidney cyclophorase gels contain TPN as well as DPN. For example, a preparation of the kidney enzyme was found to

contain 3.7 mg. of DPN/g. dry weight, and 1.0 mg. of TPN. Similarly the values for a liver preparation were 1.6 mg. of DPN and 1.1 mg. of TPN/g. dry weight. TPN like DPN is firmly bound in the cyclophorase gel and cannot be removed by exhaustive washing of the gel with 0.9% potassium chloride. The same pattern of destruction obtains for TPN as for DPN when the cyclophorase gel is incubated at 38°C.

DISCUSSION

The above data confirm that a fundamental difference exists between the oxidases of the mitochondrial cyclophorase complex and their classical counterparts with respect to the requirement for the addition of pyridine nucleotide. The possibility that the cyclophorase gel already contains free pyridine nucleotide is excluded (a) since the gel has been exhaustively washed, (b) since free pyridine nucleotide is rapidly destroyed by some enzymatic process which goes on in the gel, and (c) since tests show that pyridine nucleotide when added to the gel can be recovered by washing the gel quickly at 0° to prevent destruction. There is however a considerable amount of bound DPN and TPN in both rabbit liver and kidney cyclophorase gel. This bound form is much more stable than added pyridine nucleotide. Recently Pardee and Potter (10) have shown that the decline in the capacity of rat liver homogenates to carry on oxidative phosphorylation parallels the decline in bound DPN.

ACKNOWLEDGMENTS

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SUMMARY

The lactic, malic, citric, β -hydroxybutyric, glutamic, proline, and α -ketoglutaric oxidases in a freshly prepared cyclophorase gel work maximally in complete absence of added pyridine nucleotide. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) are both present, firmly bound to the gel of rabbit kidney and liver cyclophorase.

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Studies on the Cyclophorase System. XI. The Effect of Various Treatments on the Requirement for Pyridine Nucleotide¹

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INTRODUCTION

In a previous communication (1), data were presented which showed that in a freshly prepared cyclophorase gel the lactic, malic, citric, β -hydroxybutyric, α -ketoglutaric, proline, and glutamic oxidases worked maximally in the absence of added pyridine nucleotide. However, a freshly prepared cyclophorase gel was found to contain a very considerable amount of bound pyridine nucleotide. The present communication contains evidence that when cyclophorase gels are treated in a variety of ways, the above seven oxidases require the addition of pyridine nucleotide for full activity.

EXPERIMENTAL

All essential experimental details are given either in the appropriate section of the previous communication by Huennekens and Green (1) or in the legends for the tables and figures of the present communication.

RESULTS

Aging of Cyclophorase Gel

Preparations of cyclophorase gel which have been allowed to age at 0°C. for a period of several days are indistinguishable from fresh preparations with respect to the initial velocity with which they catalyze

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the oxidation of intermediates of the citric acid cycle or substances which give rise to these intermediates. As shown in Table I, diphosphopyridine nucleotide (DPN) does not influence the rate of oxidation of α -ketoglutarate, citrate, or glutamate for the first 30 min. Thereafter, the rate in the absence of DPN declines steadily, whilst the rate in the presence of DPN is maintained. As the aging at 0° is continued, the decline in the rate without DPN is observed almost as soon as the first measurements are made. Thus at the end of a 6-day exposure, the oxidation of citrate, glutamate, and α -ketoglutarate is not maximal in the absence of DPN even for the first recorded 10-min. period. There is some individual variation for different oxidases. Thus, in the case of this particular experiment, the proline oxidase behaved at the end of a 4-day exposure more or less like the citric, α -ketoglutaric, and glutamic oxidases at the end of a 6-day exposure. It should be pointed out that the time scale for the aging of cyclophorase gels varies from preparation to preparation.

TABLE I

*Effect of DPN on Oxidations Catalyzed by Kidney
Cyclophorase Gel Aged for 4 Days at 0°*

Each manometer vessel contained 1 ml. of R_3K^a (kept 4 days at 0°), 0.3 ml. of 0.01 *M* adenylic acid, 0.2 ml. of 0.1 *M* phosphate buffer of pH 7.3, 0.2 ml. of 0.02 *M* $MgCl_2$, 0.2 ml. of 0.2% cocarboxylase, 0.2 ml. of 1% cytochrome *c* and 0.3 ml. of 0.1 *M* substrate. Alternative manometer cups contained 0.5 ml. of 1% DPN (55% purity). Final volume 3.0 ml., alkali in center well, air in gas space, 38°. All values are corrected for by the appropriate blanks without substrate. Measurements were begun after a 5-min. equilibration period.

| Period of measurement | Oxygen absorbed, μ l. | | | | | | | |
|-----------------------|---------------------------|----------|-------------|----------|-------------|----------|-------------|----------|
| | α -Ketoglutarate | | Glutamate | | Citrate | | Proline | |
| | Without DPN | With DPN | Without DPN | With DPN | Without DPN | With DPN | Without DPN | With DPN |
| <i>min.</i> | | | | | | | | |
| 0-10 | 145 | 99 | 130 | 110 | 112 | 100 | 39 | 79 |
| 10-20 | 138 | 111 | 124 | 127 | 138 | 132 | 18 | 78 |
| 20-30 | 120 | 111 | 111 | 144 | 140 | 159 | 16 | 85 |
| 30-40 | 64 | 131 | 59 | 125 | 131 | 156 | 16 | 62 |
| 40-50 | 28 | 124 | 34 | 99 | 45 | 155 | 10 | 54 |
| 50-60 | 15 | 116 | 20 | 78 | 19 | 143 | 10 | 50 |
| 60-90 | 16 | 193 | 25 | 139 | 27 | 222 | 19 | 110 |

^a R_3K indicates a thrice-washed cyclophorase residue of rabbit kidney.

Other oxidases present in aged preparations of cyclophorase gel also show this requirement for DPN. Table II contains data for the lactic, malic, and β -hydroxybutyric oxidases in a kidney gel aged for 6 days at 0°.

It is to be noted that a very high level of DPN was used in these experiments. The reason for this high concentration will be considered in a later section of this communication. Furthermore, DPN was used even in cases where the particular oxidase is known to be specific for triphosphopyridine nucleotide (TPN), *e.g.*, the citric oxidase. In a cyclophorase gel, DPN and TPN are equivalent by virtue of the presence of an enzyme system which converts one into the other. The properties of this interconversion system will be considered in another communication.

TABLE II

Effect of DPN on Oxidation of Lactate, Malate, and β -Hydroxybutyrate Catalyzed by Aged Cyclophorase Gel

Details as in Table I, except that R₃K was 6 days old. The amount of substrate added was 0.1 ml. of the 1 M neutral solution of each of the DL-acids.

| Period of measurement | Oxygen absorbed, μ . | | | | | |
|-----------------------|--------------------------|----------|-------------|----------|--------------------------|----------|
| | Lactate | | Malate | | β -Hydroxybutyrate | |
| | Without DPN | With DPN | Without DPN | With DPN | Without DPN | With DPN |
| <i>min.</i> | | | | | | |
| 0-30 | 77 | 289 | 118 | 273 | 0 | 155 |
| 30-60 | 31 | 187 | 48 | 132 | | |

Since DPN can give rise to adenylic acid by enzymatic hydrolysis, the possibility has to be considered that the effect of DPN could be duplicated merely by increasing the initial level of adenylic acid. Experiment has shown that the decline in the absence of DPN cannot be arrested by increasing the initial level of adenylic acid from 3 to 8 μ moles.

Treatments and Reagents Which Split Out Pyridine Nucleotide

Several physical treatments, *e.g.*, freezing and thawing, exposure to deionized water or high salt concentration, and chemical reagents, *e.g.*, capryl alcohol, arsenite, gramicidin, and 2,4-dinitrophenol have been

found to depress if not abolish either the oxidative activity of cyclophorase oxidases or the accompanying oxidative phosphorylation or both (2-4). All these treatments or reagents can readily be shown to split out DPN from the cyclophorase gel in varying amounts⁴ (cf. Table III), and to a comparable degree render the oxidases in the gel so treated dependent upon the addition of DPN for full activity (cf. Table IV). The malic oxidase has been selected to illustrate this particular

TABLE III

Release of Endogenous DPN

Each sample consisted of 4.0 ml. of R_3L , 1.0 ml. of 0.33 *M* fluoride, other components as indicated. All samples (except Nos. 3 and 4) were kept at 0° for 10 min. The samples were washed three times with 0.9% potassium chloride (exceptions: No. 2 with 0.12 *M* sucrose and No. 11 with deionized water), and a 1 ml. aliquot of each was heat coagulated and analyzed for residual DPN by the customary method.

| Number | Reagent and treatment | Residual DPN <i>μg.</i> |
|--------|--------------------------------------|----------------------------|
| 1 | None | 140 |
| 2 | Washed with 0.12 <i>M</i> sucrose | 110 |
| 3 | 38°, with 0.01 <i>M</i> fluoride | 105 |
| 4 | 38°, fluoride omitted | 63 |
| 5 | 2,4-Dinitrophenol (0.0017 <i>M</i>) | 70 |
| 6 | Sodium chloride (0.5 <i>M</i>) | 14 |
| 7 | Gramicidin (saturated solution) | 14 |
| 8 | Arsenite (0.017 <i>M</i>) | 14 |
| 9 | Capryl alcohol (saturated solution) | 14 |
| 10 | Freezing and thawing | about 5 |
| 11 | Washed with deionized water | about 0 |

point since it is the most readily dissociable of all the oxidases under study. Similar results obtain for most of the other oxidases. The degree to which DPN is split out from the gel by a particular reagent is a function of the concentration of the reagent as illustrated in Table V for arsenite at various concentrations.

Table VI summarizes the extent to which the lactic, malic, citric, α -ketoglutaric, proline, glutamic, and β -hydroxybutyric oxidases are

⁴ In this paper a conjugated oxidase is defined as one in which the protein moiety (apoenzyme) and the prosthetic group (coenzyme) are firmly bound together. Splitting or resolution of the oxidase refers to the dissociation of the two components. A split oxidase is an apooxidase which requires added coenzyme for activity.

TABLE IV
Splitting of the Malic Oxidase of Cyclophorase Gel

Each Thunberg tube contained 1.0 ml. of R₃L (exception: in Expt. 8, acetone precipitate was suspended in 10 vol. of 0.9% KCl), 1.0 ml. of 0.1 M DL-malate, 1.0 ml. of 2-amino-2-methyl-1,3-propanediol buffer of pH 9.5, 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol, and 250 µg. of DPN where indicated.

The reagents were added directly in Expts. 1-4; in Expts. 5-12 the enzyme was washed twice with 0.9% KCl following the particular treatment.

| Number | Reagent or treatment | Temperature of exposure | Time of exposure | Velocity (sec. ⁻¹ × 10 ³) | | Split of malic oxidase ^a |
|--------|--|-------------------------|------------------|--|-------------|-------------------------------------|
| | | | | With DPN | Without DPN | |
| | | °C. | min. | | | per cent |
| 1 | Capryl alcohol (saturated solution) | 25 | 10 | 40 | 0 | 100 |
| 2 | Arsenite (0.017 M) | 25 | 10 | 14.3 | 6.9 | 52 |
| 3 | Gramicidin (0.017%) | 0 | 10 | 52.8 | 22.2 | 58 |
| 4 | 2,4-Dinitrophenol (1.7 × 10 ⁻³ M) | 0 | 10 | 45.4 | 18.2 | 60 |
| 5 | Sodium chloride (0.5 M) | 0 | 10 | 22.7 | 12.5 | 45 |
| 6 | Sodium chloride (0.5 M) | 38 | 10 | 8.7 | 0 | 100 |
| 7 | Ammonium sulfate (80% saturated) | 25 | 10 | 9.8 | 0 | 100 |
| 8 | Acetone precipitation | 0 | — | 6.5 | 0 | 100 |
| 9 | Deionized water | 0 | 10 | 5.1 | 0 | 100 |
| 10 | Freezing and thawing (4 times) | 0 | — | 23.2 | 7.7 | 67 |
| 11 | Aging | 0 | 1 day | 40.0 | 19.6 | 51 |
| 12 | Incubation | 38 | 5 | 22.2 | 14.7 | 34 |

^a Percentage splitting is defined as that proportion of the total oxidase which is in the split state. It is calculated as the ratio of the reaction velocities

$$\frac{(\text{with DPN} - \text{without DPN})}{(\text{with DPN})} \times 100.$$

split⁵ following exposure of the cyclophorase gel to a variety of treatments. There is great variability in the time necessary for any one oxidase to undergo splitting during exposure to the reagents. Some of the complicating factors are considered under *Discussion*. The purpose of Table VI is to summarize the general trend of these experiments rather than to provide exact conditions for splitting individual oxidases.

⁵ The enzymatic test system determines intact DPN remaining in the cyclophorase gel. Splitting out of DPN is intended to convey only the idea that the original, bound DPN is no longer present, as such, in the preparation. In a system where "free" DPN is rapidly broken down by pyrophosphatase and nucleosidase activities (discussed in the previous paper), we have not investigated the fate of the split out DPN.

Capryl alcohol has proved to be one of the most effective reagents for completely splitting the pyridinoprotein oxidases of the cyclophorase complex. The cyclophorase gel is saturated with capryl alcohol and kept at 0° during the entire exposure period which may vary from 1 to 24 hr. depending upon the enzyme. Usually the gel is washed with cold 0.9% KCl at the end of the exposure and before testing for enzymic activity. The treatment with capryl alcohol destroys the β -hydroxybutyric oxidase. The proline oxidase is only partially split under conditions which lead to complete splitting of the other oxidases. With the proline oxidase, increased dissociation of the coenzyme is matched by destruction of the apoöxidase; hence it will appear as if there is less splitting at the end of a 5-day exposure than at the end of 1 day. All the evidence points to a conjugated pyridinoprotein being much more stable than its apoöxidase. In consequence there has to be careful selection of the exposure time which will strike a proper balance between the extent of splitting of the conjugated oxidase and the extent of destruction of the apoöxidase.

Treatment of the cyclophorase gel with acetone at -5° yields a preparation containing completely dissociated lactic, malic, glutamic, and citric oxidases but only a partially split α -ketoglutaric oxidase. The β -hydroxybutyric and proline oxidases are largely destroyed by the acetone treatment. In general, acetone treatment leads to considerable loss of activity of all the oxidases and for that reason is not too satisfactory.

Exposure of the cyclophorase gel to deionized water at 0° for 24 hr. has proved to be one of the mildest procedures for resolving some of the oxidases which are unstable either as conjugated or split enzymes. Thus the β -hydroxybutyric oxidase is almost completely split by this

TABLE V

Effect of Exposure to Arsenite on Level of Endogenous DPN

For details see legend of Table IV. No fluoride was added to any of the samples. All samples were kept at 0° for 10 min. before removing the reagent by three washings.

| Arsenite concentration moles/l. | DPN remaining $\mu\text{g.}/\text{ml.}$ |
|------------------------------------|--|
| 0 | 130 |
| 1×10^{-5} | 130 |
| 1×10^{-4} | 40 |
| 1×10^{-3} | 29 |
| 2×10^{-3} | 24 |
| 2×10^{-2} | 18 |

TABLE VI *Splitting of Oxidases of Cyclophorase Gel by Various Procedures*

| Treatment or reagent exposed to | Time | Temperature °C. | Lactic ^a split per cent | Malic ^a split per cent | Citric ^b split per cent | α -Ketoglu- taric ^b split per cent | Proline ^a split per cent | Glutamic ^a split per cent | β -Hydroxy- butyric ^a split per cent |
|--|----------|--------------------|--|---|--|---|---|--|--|
| 1. Capryl alcohol (satd. solution) | variable | 0 | 94 | 99 | 100 | 87 | 66 | 100 | inactivated |
| 2. Arsenite (0.17 M) | 24 hr. | 0 | 0 | 95 | inactivated | 45 | 0 | 87 | inactivated |
| 3. 2,4-Dinitrophenol (0.01 M) + gramicidin (satd. aq. soln.) | 24 hr. | 0 | 56 | 66 | 47 | 0 | 30 | 33 | inactivated |
| 4. Deionized water | 24 hr. | 0 | 25 | 84 | 92 | 6 | 61 | 72 | 100 |
| 5. Sodium chloride (75% satd.) | 15 min. | 0 | 0 | 70 | 84 | 0 | 33 | 58 | — |
| 6. Ammonium sulfate (80% satd.) | 9 days | 0 | 0 | 100 | 100 | 0 | 0 | 100 | — |
| 7. Precipitated with acetone | 10 min. | -5 | 100 | 100 | 100 | 91 | — | 100 | — |

^a Reactivated with DPN.^b Reactivated with TPN.

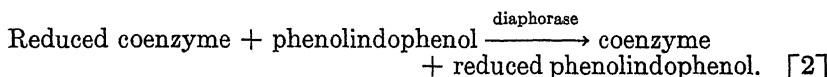
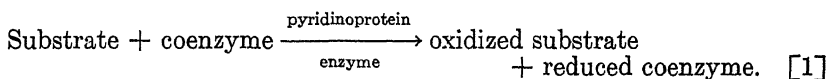
Details for Various Treatments

1. Capryl alcohol was removed by washing the gel at end of exposure period. The lactic, malic, and glutamic oxidases were exposed for 1 hr.; the citric and proline oxidases for 4 hr., and the α -ketoglutaric oxidase for 24 hr. Kidney cyclophorase gel.
2. Liver cyclophorase gel (R_3L) after exposure to the reagent was washed with 20 vol. of 0.9% potassium chloride solution before testing.
3. Details as for 2.
4. Details as for 2.
5. After exposure the gel (R_3L) was washed free of salt twice with 20 vol. of 0.9% potassium chloride before testing.
6. Details as for 5.
7. Kidney cyclophorase gel was precipitated with 5 vol. of acetone at -5° . The precipitate was washed with acetone and then air dried. The acetone powder was resuspended evenly in 10 vol. of 0.9% potassium chloride and the whole suspension tested.

treatment while the proline oxidase, the most difficult of all to resolve by other procedures, can be split to the extent of 60% or more.

In testing for the presence of split oxidases, the conditions are identical with those used in the test for conjugated oxidase except in the cases of the lactic and malic oxidases. The conjugated lactic and malic oxidases work maximally at about pH 7.0 whereas the corresponding apoöxidases are almost inactive at this pH in the absence of a fixative for the product of reaction. The tests for the apomalic and apolactic oxidases were carried out routinely at pH 8.5–9.5 at which pH no fixative has to be employed.

In the anaerobic Thunberg method the rate of reduction of 2,6-dichlorophenolindophenol is used as the measure of the activity of the various oxidases. This process involves two consecutive reactions each of which is catalyzed by a different enzyme:



Unless diaphorase is in excess, the over-all rate of reduction is not a measure of the activity of the apoöxidase. Whenever tests have been applied it has been found that diaphorase is present in large excess. Potter (5), in his studies of rat liver homogenates, came to a similar conclusion about the excess of diaphorase. Addition of diaphorase to the cyclophorase gel did not increase the rate of reduction of 2,6-dichlorophenolindophenol. Furthermore, diaphorase did not appear to be split to any significant extent by the various procedures which were used to split the pyridinoprotein oxidases of the cyclophorase complex.

Saturation Level of Pyridine Nucleotide for Apoöxidases

Experiments were designed to determine the level of pyridine nucleotide at which the various apoöxidases function at maximum velocity. For any given oxidase this level was found to fluctuate widely in different experiments. Eventually this variation was referred to the destruction of DPN which proceeded at different velocities in different preparations of the cyclophorase gel. Figure 1 graphically describes a

typical experiment. The velocity of reaction as a function of the concentration of DPN was measured in three series of experiments which differed in the following respects. In the first series (A), the coenzyme was placed in the side arm of the Thunberg tube with the indophenol solution, and mixed with the enzyme gel at the beginning of the experiment. In the second series (B), the coenzyme was incubated with the enzyme gel for 2 min. at 38° before the indophenol solution was tipped in. Finally in the third series (C), the coenzyme was incubated with the

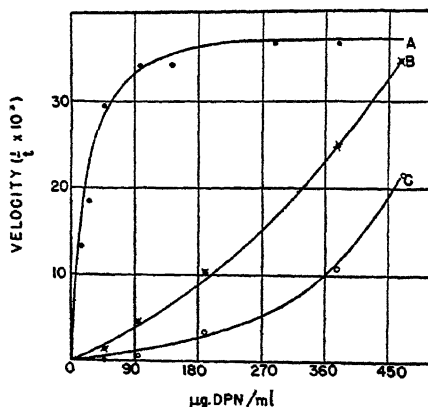


FIG. 1. Velocity as a function of the concentration of DPN after varying times of preincubation of the coenzyme with the cyclophorase gel. A, 0 min.; B, 2 min.; C, 5 min. These experiments were carried out anaerobically in Thunberg tubes at 38°. Each tube contained 0.5 ml. of R₂K previously suspended in one-tenth the volume of 0.1 M arsenite, 0.4 ml. of 1 M amino-1,3-propanediol buffer of pH 9.2, 0.04 ml. of capryl alcohol, 0.5 ml. of 0.1% 2,6-dichlorophenolindophenol, 0.2 ml. of 1 M DL-malate, DPN as indicated, and water to a final volume of 3.4 ml.

enzyme gel for 5 min. before the start of the experiment. The same limiting velocity was attained in all three series, but there was a progressive increase in the level of pyridine nucleotide at which maximum velocity was attained. It is apparent from the curves that 0.5 ml. of the arsenite-capryl-treated kidney gel can destroy about 1000 μg. of DPN in 5 min. at 38° under anaerobic conditions. There is, therefore, no possibility of determining accurately the saturation level of pyridine nucleotide for any of the apooxidases of the treated cyclophorase gel.

DISCUSSION

The evidence presented in this and the previous communication (1) indicates that the principal oxidases of the cyclophorase complex occur as conjugated pyridinoprotein enzymes and that dissociation into apoxidases comes about only as a result of partial denaturation or modification of the cyclophorase complex. There is, however, an alternative interpretation, the merits of which will have to be assessed before excluding the possibility that at least some of the oxidases occur as dissociated pyridinoproteins in the undenatured cyclophorase complex. If one assumes that the cyclophorase complex is associated with a structural unit having a membrane separating an internal fluid from the external medium⁶ and that the coenzyme present within the interior of the structural unit is free and in solution, it would be in order to postulate that all the pyridinoprotein enzymes of the cyclophorase complex are fully dissociated and that the amount of free DPN and TPN locally present is enough to saturate all the enzymes. Hence, there would be no effect of added DPN or TPN. When the unit is disrupted the internal coenzymes are dispersed and diluted to the point where the apoenzymes are essentially inactive unless supplemented with additional coenzyme.

There are, however, many observations which cannot be reconciled with this hypothesis. How can one explain that following a given treatment (*e.g.*, exposure to high concentrations of ammonium sulfate), some of the oxidases are found to be fully split, whereas the rest are found essentially in the unsplit condition? One would anticipate that the oxidases would behave uniformly if they were all originally dissociated and dependent upon the same pool of unbound coenzyme, particularly in view of the fact that all apoxidases are known to be saturated at approximately the same level of added pyridine nucleotide. If the coenzyme in the interior of the mitochondrial unit were free and diffusible there is no satisfactory explanation for the facts that (a) the pyridine nucleotide content of the cyclophorase gel remains unchanged despite dilution of the gel with as much as 100,000 vol. of salt solution, and (b) the behavior of endogenous pyridine nucleotide is entirely different from that of externally-added pyridine nucleotide.

⁶ J. Harman (6) in this laboratory has presented evidence that the mitochondrial unit is more in the nature of a gelatinous fiber than an osmotic system like the red blood corpuscle. Dalton (7) and his colleagues, however, favor the view that mitochondria behave like miniature cells with a limiting membrane and a liquid internal medium.

Several of the oxidases of the cyclophorase gel (*e.g.*, the malic and glutamic oxidases) are completely inhibited by reagents such as capryl alcohol. However, when the same treated gel is supplemented with pyridine nucleotide, this inhibitory effect disappears. Such a result cannot be explained on the basis that this reagent merely disperses free pyridine nucleotide previously concentrated within the mitochondria. Other oxidases of the cyclophorase gel when exposed to these reagents for the same short period suffer little or no inhibition. Furthermore, the malic oxidase after this treatment is no longer active at the same pH as the malic oxidase of the untreated cyclophorase gel. Again this result is only compatible with the assumption that the state of conjugation of the oxidase has undergone some alteration in consequence of the exposure of the oxidase to capryl alcohol.

Perhaps the most cogent bit of evidence is that by procedures which lead to the dissolution of the structural unit of the cyclophorase, one may readily prepare conjugated α -ketoglutaric, lactic, malic, glutamic, citric, and proline oxidases [cf. Huennekens and Green (1)]. By using the expedient of prolonged exposure to the action of the Waring Blendor, preparations can readily be made in which the structural unit is no longer recognizable although the component oxidases are still fully conjugated.

The mechanism by which the pyridine nucleotides are split out of the cyclophorase gel and by which certain oxidases are split following certain treatments, is still somewhat obscure. Our own interpretation is that the rupture of the protein-pyridine nucleotide linkage may involve two separate processes:

- a) The splitting reagent may have a higher affinity for the apoenzyme than does the pyridine nucleotide.
- b) The splitting reagent or physical treatment may reorient the gel surface in such a way that a splitting enzyme may now effect the actual cleavage of the bond between protein and pyridine nucleotide.

In view of the fact that autolytic splitting is proceeding all the time, it is important to know how much of the splitting of any particular oxidase is due to the introduction of the reagent and how much would have taken place in the absence of the reagent. In general, the reagents employed in the experiments summarized in Tables III and IV operate far more rapidly than does the spontaneous autolytic process. However, there are a few borderline cases in which there is uncertainty on this score.

An interesting point of difference between the conjugated oxidases of cyclophorase and the dissociated oxidases derived from cyclophorase relates to their solubility. All of the latter category with the exception of the α -ketoglutaric and proline oxidases have been prepared as soluble oxidases, whereas all of the former exist exclusively in particulate form. By drastic procedures such as precipitation with acetone we have found that it is possible to render most of the cyclophorase oxidases soluble. Thus, a pattern of progressive denaturation can be recognized for any of the cyclophorase oxidases. With mild procedures the particulate character of the oxidase is preserved but the coenzyme is dissociated. With more drastic procedures the split oxidase is brought into solution.

The Coris in 1945 (8-10) made the important discovery that a highly purified crystalline preparation of the aldotriosephosphate dehydrogenase of rabbit skeletal muscle contained DPN firmly linked to the protein, presumably in stoichiometric amount. In this enzyme the linkage of DPN and protein was sufficiently strong to prevent dissociation during the course of purification. There is in this respect resemblance to the proline and α -ketoglutaric oxidases.

ACKNOWLEDGMENTS

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SUMMARY

After cyclophorase gels have been (a) aged at 0° for some hours, or (b) exposed to alternate freezing and thawing, deionized water, strong salt solutions, 2,4-dinitrophenol, arsenite, acetone, and capryl alcohol, various oxidases which previously showed no requirement for pyridine nucleotide are now partially or completely dependent for activity upon the addition of pyridine nucleotide. Evidence is presented that these treatments lead to the splitting out of bound pyridine nucleotide and to the transition from conjugated to dissociated pyridinoprotein enzymes.

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The Nature of Certain Impurities Present in Blood Group A Substance Preparations Derived from Hog Gastric Mucosa¹

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INTRODUCTION AND RESULTS

In an earlier communication (1) it was shown that A-substance preparations derived from hog gastric mucosa may be contaminated with substances absorbing maximally at 260 m μ . It has now been found that if a series of A-substance preparations obtained from hog gastric mucin (2,3), and absorbing maximally at 260 m μ , are arranged in order of increasing extinction at 260 m μ , the same order is maintained with respect to their phosphorus contents (cf. Table I). Furthermore for seventeen of the twenty preparations examined the ratio of % P \times 10³ / $E_{1\text{cm}}^{1\%}$ at 260 m μ (P/E ratio) was found to be 30 ± 8 .³ In view of the fact that nucleic acids and related compounds absorb maximally at 260 m μ (4,5) and that the P/E ratios for pneumococcus nucleic acid (6,7), tobacco mosaic virus nucleic acid (8,9), and adenylic acid (10) are 19, 30, and 20, respectively, it may be concluded that the contaminants previously designated as the "260 m μ component" (1,11) are nucleic acids or nucleoproteins characterized by a P/E ratio of 33 ± 2 ⁴ and

¹ Supported in part by a grant from the U. S. Public Health Service.

² Contribution No. 1337.

³ The P/E ratios of preparations R20-F2, C-135, and R17-F4 were not included in this average.

⁴ In order to arrive at a reasonably accurate value for P/E, all of the data given in Table I for the R-series of preparations was subjected to a least squares treatment and those ten sets of data which showed minimum deviation from the mean were selected for a second least squares treatment to establish the relation between % P and $E_{1\text{cm}}^{1\%}$ at 260 m μ . The relations obtained were: % P = $0.033 (E_{1\text{cm}}^{1\%} \text{ at } 260 \text{ m}\mu) - 0.01$ and $E_{1\text{cm}}^{1\%} \text{ at } 260 \text{ m}\mu = 30 (\% \text{ P}) + 0.30$. From these equations it follows that the P/E ratio is 33 and that, of the total extinction at 260 m μ , 0.3 is due to nonspecific absorption.

TABLE I
Properties of A-Substance Preparations

| Preparation | Relative activity ^a | $E_{1cm}^{1\%b}$ | P | P/E ratio ^c | S ^d | Relative viscosity | | pH of solution ^e | Gel formation ^f | |
|---------------------|--------------------------------|------------------|-------|------------------------|----------------|--------------------|------------|-----------------------------|----------------------------|----------------------|
| | | | | | | 0.2% at 25 | 0.5% at 37 | | FeCl ₃ | Hg(OAc) ₂ |
| R20-F2 | 6 | 0.87 | 0.01 | 12 | 0.28 | 1.55 | 2.67 | 4.3 | — | ++ |
| R18-F10 | 5 | 0.91 | 0.025 | 28 | 0.19 | 1.41 | 1.97 | 4.9 | — | ± |
| R20-F1 | 5 | 1.17 | 0.03 | 26 | 0.21 | 1.32 | 1.84 | 4.1 | — | — |
| R18-F11 | 5 | 1.20 | 0.035 | 29 | 0.23 | 1.42 | 1.97 | 4.2 | — | ± |
| R18-F7 | | 1.79 | 0.04 | 22 | 0.22 | 1.42 | 2.19 | 5.9 | — | + |
| R18-F9C | 4 | 1.97 | 0.06 | 30 ^h | | 1.35 | 1.92 | 4.1 | — | — |
| R18-F13 | 4 | 2.15 | 0.06 | 29 ^h | | 1.29 | 1.73 | 5.2 | — | — |
| R18-F8A | | 2.52 | 0.08 | 32 ^h | 0.27 | 1.41 | 2.14 | | | |
| R18-F2 | | 3.50 | 0.10 | 28 ^h | 0.24 | 1.47 | 2.34 | 6.2 | + | ++ |
| R18-F12 | 4 | 3.80 | 0.12 | 32 ^h | | 1.39 | 2.10 | 6.0 | ++ | ++ |
| R18-F6A | 2.5 | 5.05 | 0.15 | 30 ^h | 0.21 | 1.50 | 2.47 | 6.1 | ++ | ++ |
| R18-F5 | | 5.20 | 0.16 | 31 ^h | 0.28 | 1.45 | 2.26 | 3.2 | — | ++ |
| R18-F1A | 1 | 8.03 | 0.25 | 31 ^h | 0.26 | 1.51 | 2.46 | 6.0 | ++ | ++ |
| R17-F3 | | 9.63 | 0.32 | 33 ^h | | 1.45 | 2.31 | 4.9 | ++ | ++ |
| C-135 | 1 | 22.8 | 0.91 | 40 | 0.36 | 1.36 | 2.32 | 4.8 | ++ | + |
| R17-F2 | 1 | 36.4 | 1.20 | 33 ^h | 0.67 | 1.33 | 1.95 | 4.6 | ++ | + |
| R17-F4 ^g | 0.1 | 36.4 | 1.86 | 51 | | | | | | |
| R18-F3 | 0.5 | 68.1 | 1.28 | 19 | | | | | | |
| C-52 ^h | 2(1.5) | 11.6 | 0.36 | 31 | | 1.75 | 3.10 | | ++ | + |
| C-47 ⁱ | 4.5(1) | 0.8 | 0.03 | 38 | | 1.21 | 1.50 | | — | — |
| C-Z1 ^j | | 4.50 | 0.15 | 33 | | 1.43 | 2.18 | | | |

^a Based upon both inhibition of hemolysis and inhibition of isoagglutination with preparation C-135 (centrifuged Wilson mucin suspension) as the reference standard assumed to have unit activity. In those cases where parallel results were not obtained in both tests the value given in parentheses is the inhibition of isoagglutination value and the other the inhibition of hemolysis value. For further details see Refs. (1-3,13).

^b At 260 mμ.

^c % P × 10³ / $E_{1cm}^{1\%}$ at 260 mμ.

^d Present as total hydrolyzable sulfate.

^e pH of 1% solution in water.

^f No gel, —; questionable gel, ±; thin gel, +; firm gel, ++.

^g Maximum at 270 mμ, $E_{1cm}^{1\%}$ = 38.

^h Prepared by the Morgan phenol procedure (18).

ⁱ A degraded A-substance preparation obtained by the procedure of Meyer, Smyth, and Palmer (14).

^j Prepared by the procedure of Zittle (23).

^k Values used for the evaluation of P/E ratio of 33 ± 2.

that these substances are present in many A-substance preparations derived from hog gastric mucin (1,12).

The solids present in a centrifuged solution of hog gastric mucin (cf. Table I, preparation C-135) have a P/E ratio of 40. However, if this solution is dialyzed, 26% of the phosphorus is lost and a P/E ratio of 29 is obtained. Thus practically all of the nondialyzable phosphorus in hog gastric mucin appears to be present as nucleic acids or nucleoproteins. Of the various methods that have been described for the isolation of A-substance from hog gastric mucin [cf. (2,3,13)] the only one that has given an unmodified A-substance preparation substantially free⁵ of nucleic acids or nucleoproteins is the procedure based upon ethanol fractionation and electrodecantation (2) followed by precipitation at pH 3.0 in an aqueous solution of minimum ionic strength (3). A preparation (cf. Table I, preparation C-47) obtained by the procedure of Meyer, Smyth, and Palmer (14), while substantially free of nucleic acids, was found to be degraded (1).

From information now available it can be concluded that A-substance and H-substance (formerly designated as O-substance) do not contain nucleic acids or phosphorus, and if A-substance preparations are found to absorb specifically at 260 $m\mu$ and to contain nondialyzable phosphorus, the extent of contamination of these preparations by nucleic acids can be estimated either from the extinction coefficient at 260 $m\mu$ or the nondialyzable phosphorus content, or both. For example, a preparation containing 1% of phosphorus (15) would appear to contain at least 10% of nucleic acids.

Sulfur-containing polysaccharides are known to be present in hog gastric mucosa (16), and acidic polysaccharides containing 3.1–4.7% sulfate sulfur have been isolated from hog gastric mucin (14). It is therefore reasonable to expect that the solids of a centrifuged mucin solution with a total sulfate-sulfur content of 0.36% (cf. Table I, preparation C-135) might contain 8–12% of such acidic polysaccharides. From the data given in Table I it is clear that although the more active preparations, R20-F2, R18-F10, R20-F1, and R18-F11, contain less sulfate sulfur than mucin, the possibility still exists that they may be contam-

⁵ Preparation R20-F2, the most active unmodified A-substance preparation which we have obtained from hog gastric mucin, has a phosphorus content of 0.01%. Assuming a reasonable value for the phosphorus content of the nucleic acids, *i.e.*, 9%, it is obvious that the amount of nucleic acid present in this preparation is of the order of 0.1%.

inated with 6–9% of acidic polysaccharides. In this connection it is of interest to note that the most active preparation (R20–F2) was found to contain approximately 15% of a component migrating anodically at a rate significantly faster than the main component (3). It would appear from the sulfate-sulfur content, the phosphorus content, and the extinction value that both nucleic acids and acidic polysaccharides of the sulfuric acid half-ester type tend to accumulate in the precipitate obtained from a 40% aqueous ethanol solution of centrifuged gastric mucin (preparation R17–F2).

In order to compare the relative viscosities of 0.2% solutions of A-substance preparations in 0.9% saline at 25° (17) with those of 0.5% solutions in 0.9% saline at 37° (18), the relative viscosities of all of the A-substance preparations given in Table I except two were determined under the above conditions. It was found, with a least squares treatment, that the relative viscosities of 0.2% solutions in 0.9% saline at 25° (Y) were related to the relative viscosities of 0.5% solutions in 0.9% saline at 37° (X) by the equation $Y = 0.30 X + 0.76$. Excluding one set of values (preparation C-135) which was clearly out of line, the maximum deviation between experimental and calculated values was about 3%.

Morgan and King (18) have reported that solutions of their more active A-substance preparations derived from hog gastric mucin were always more viscous than solutions of their less active preparations, and that degradation of A-substance is accompanied by a substantial decrease in the viscosity of the solutions. While it is true that the relative viscosities of solutions of the degraded preparation C-47 are the lowest of all of those given in Table I, it is also true that solutions of undegraded preparations of nearly equivalent activities have widely differing viscosities, and that solutions of less active undegraded preparations frequently have greater viscosities than those of more active ones. It appears that impurities present in many A-substance preparations are in large part responsible for the behavior noted above.

It has been reported (18) that aqueous solutions of A-substance preparations derived from hog gastric mucin form firm gels when treated with mercuric acetate but that no gelation is observable on the addition of ferric chloride or lead acetate. The ability of various metallic salts to cause the gelation of 1% aqueous solutions of a number of A-substance

preparations was investigated, and the data so obtained are given in Table I. With but one exception⁶ the addition of ferric chloride caused the gelation of 1% solutions of all A-substance preparations containing more than 0.09% phosphorus and having an $E_{1\text{cm}}^{1\%}$ at 260 m μ greater than 3.0. Thus, 1% solutions of those A-substance preparations which contain more than approximately 1% of nucleic acids will in general form gels when treated with ferric chloride.

Gelation upon the addition of mercuric acetate appears to be associated with the presence of the so-called acid-insoluble form of A-substance (3) since all of the preparations giving gels with mercuric acetate are those obtained as the insoluble fractions either by precipitation with ethanol, electrodecantation, or acidification to pH 3 in aqueous solutions of minimum ionic strength (2,3). Although all available evidence indicates that gelation with mercuric acetate is a characteristic property of the acid-insoluble form of A-substance (compare, for example, the behavior of preparations R20-F2 and R20-F1), it should be pointed out that a homogeneous preparation of the acid-insoluble form has not been obtained and the possibility exists that gelation with mercuric acetate is a property of an undisclosed impurity having similar solubility characteristics.

Morgan and King (18) have stated that undegraded A-substance preparations form elastic gels with 0.05 *M* borate buffer of pH 8.5, whereas degraded preparations do not. In examining over twenty A-substance preparations of varying activity and which were judged to be undegraded on the basis of comparable activity in the isoagglutinin and hemolysis tests (13,18), not one was found to form a gel under the above conditions. The reason for the discrepancy is not known.

Traces of proteolytic enzymes present in blood group specific substance preparations could cause serious difficulties in any of the serological tests used to evaluate blood group activity. Therefore, freshly prepared centrifuged suspensions of Wilson hog gastric mucin and Viobin "40° Hog Stomach Lining" were tested for proteolytic activity. The Viobin preparation contained 4.3×10^{-4} units of pepsin, 0.6×10^{-4} units of cathepsin, and 0.13 units of gelatinase/mg. of substance, and no trypsin or papain. Wilson mucin was free of all of these enzymes.

⁶ The low pH (3.2) of an aqueous solution of this preparation (R-18-F5) may be responsible for the lack of gelation.

EXPERIMENTAL

A-Substance Preparations

The methods used for the isolation of the various A-substance preparations as well as a description of their serological activities has been given previously (1-3,13). The R-series of preparations used in this study were obtained from hog gastric mucin through the use of a procedure based upon ethanol fractionation and electrodecanation (R17 and R18 series) followed by precipitation at pH 3 in aqueous solutions of minimum ionic strength (R20 series). The extinction coefficients were determined as before (1).

Total Phosphate

The samples were digested as directed by Umbreit, Burris, and Stauffer (19) and the phosphorus determined in the colorless or pale yellow digests by the method of Fiske and SubbaRow (20).

Total Hydrolyzable Sulfate

Samples of approximately 300 mg. each were refluxed for 2 hr. with 50 ml. of 1 *N* hydrochloric acid, the hydrolysate was cooled, filtered through a fine sintered glass filter, and the sulfate determined gravimetrically as barium sulfate.

Viscosity

Solutions 0.2% and 0.5% in substance and 0.9% in sodium chloride were prepared, and measurements made with Ostwald-type viscometers using 10 ml. of solution. Relative viscosities were calculated by dividing the efflux time for the solution by the efflux time for 0.9% sodium chloride.

Gel Formation with Metallic Salts

To 1 ml. of a 1% solution of the mucin fraction in water in a small test tube was added 0.05 ml. of a 0.1 *M* solution of the metallic salt. The tubes were shaken and allowed to stand for 2-3 min. before noting the results.

Gelation Experiments with Borate Buffer

Palitsch's borate buffer of pH 8.5 was prepared by mixing equal volumes of 0.2 *M* boric acid and 0.05 *M* sodium tetraborate. The gelation experiments with this buffer were carried out according to Morgan and King (18).

Determination of Proteolytic Activities

Proteolytic activities of freshly prepared centrifuged 2% suspensions, or dilutions thereof, of Wilson hog gastric mucin and Viobin 40° hog stomach lining were determined in the following manners and the activities expressed as they are defined in the respective references. Pepsin, trypsin, papain (HCN activation), and cathepsin activ-

ities were estimated by the hemoglobin method of Anson using the phenol reagent to measure the extent of hydrolysis (21); "gelatinase" activity was determined by the method of Northrop, expressing the result in the unit $[PU]_{\text{gel}}^{1\%}$ (22).

SUMMARY

1. Nucleic acids characterized by a ratio of $\% P \times 10^3 / E_{1\text{cm}}^{1\%}$ at 260 μ of 33 have been shown to be present as impurities in many A-substance preparations derived from hog gastric mucosa.

2. The presence of sulfate sulfur in the more active A-substance preparations has been established. It is not known whether this component is characteristic of A-substance or of an impurity present in these preparations.

3. The relation between the relative viscosities of 0.2% solutions of A-substance preparations in 0.9% saline at 25° and of 0.5% solutions at 37° has been determined.

4. The gelation of aqueous solutions of A-substance preparations by ferric chloride has been shown to be a property of preparations contaminated with more than 1% of nucleic acids, whereas gelation by mercuric acetate has been associated with the presence of the so-called acid-insoluble form of A-substance in these preparations.

5. Wilson hog gastric mucin has been found to be devoid of proteolytic activity.

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The Nature of Some Fluorescing Substances Contained in a Deep-Sea Mud ¹

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INTRODUCTION

It was found earlier that some colorless polyenes such as phytofluene (1) or phytoflueneol (2) are widely spread in land plants and impart intense fluorescence to their extracts. In view of the fluorescence of mud extracts, it seemed advisable to extend our experiments to such materials. As is well-known, phytofluene in many cases accompanies carotenoid pigments in the land flora. On the other hand, it was shown by Fox and his collaborators (3) that sediments of the ocean floor contain substantial amounts of carotenoids deposited there centuries ago.

The sample studied in the present work was collected from a water depth of 216 m., and the mud depth amounted to 155–160 cm. The analyzed mud would range in age from fresh material (at the top) to some 960 years at the lowest level of the core (3).

Chromatographic resolution of our extracts confirmed the statement of Fox, Updegraff, and Novelli (3) that in deep-sea muds the hydrocarbon carotenoids by far exceed the oxygenated pigment types. In the sample described below, almost no xanthophylls were present. Among the eight or nine pigments, β -carotene was the relatively major one. Remarkable was the presence of a particularly strongly adsorbed red pigment possessing its main extinction maximum in hexane at 481 m μ .

In contrast, neither phytofluene nor similar colorless, fluorescing polyenes could be detected. The moderately intense, bluish gray fluores-

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³ Contribution No. 477 (New Series).

cence of the extracts was mainly caused by compounds of non-polyenic type whose spectral curves appear in Fig. 1. Since the stability of phytofluene *in vitro* is far inferior to that of polyenes possessing a substantially longer conjugated system, phytofluene possibly is the first to be destroyed under the conditions which prevail in deep-sea muds.

EXPERIMENTAL

The mud cores (3 and 4) were obtained off the coast of Southern California at the respective locations: lat. $32^{\circ}37.84'$ N, long. $117^{\circ}22.18'$ W; and lat. $32^{\circ}37.72'$ N, long. $117^{\circ}22.10'$ W. Mud from the top

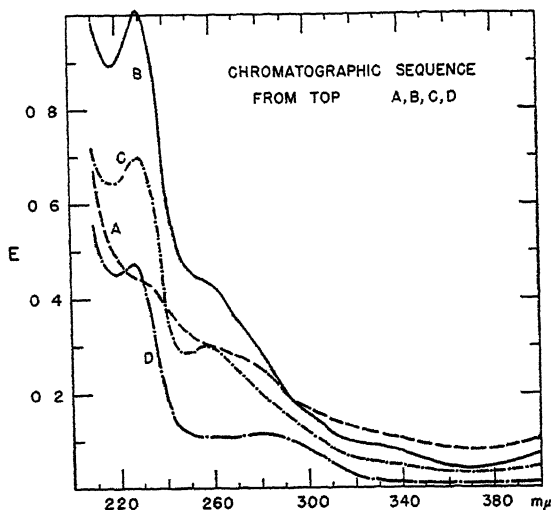


Fig. 1. Spectral curves (in hexane) of some fluorescing compounds contained in a deep-sea mud sample; *E* designates optical density.

35–40 cm. of a core yielded the analytical data listed in Table I which are based upon samples dried for 48 hr. at 100°C . under reduced pressure, giving a water-loss of about 55%.

A 15-kg. sample (wet) was stirred with methanol and upon sedimentation the supernatant liquid was siphoned off. After we had repeated these operations, the combined solutions were extracted thoroughly with petroleum ether (b. p. $60\text{--}70^{\circ}$). The remaining material was extracted four times with petroleum ether-methanol mixtures (1:1, 2:1, 3:1, and 3:1). After filtration in a basket centrifuge (diameter, 20 cm.),

TABLE I

*Analytical Data For Some Organic Constituents
of a Dried Deep-Sea Mud Sample^a*

| | % |
|---|------|
| Total organic matter (by chromic acid oxidation; ferrous ion was absent and sulfide negligible)..... | 5.46 |
| Total organic nitrogen (Kjeldahl)..... | 0.32 |
| Total lipid-soluble matter (by extraction with ethanol-ether 1:1) 0.145% (or 2.65% of total organic matter) | |
| Nonsaponifiable matter 0.066% (or 45.6% of lipid-soluble matter; soluble in petroleum ether) | |
| Organic acids 0.055% (or 37.9% of lipid-soluble matter; soluble in petroleum ether) | |
| Residue 0.024% (or 16.6% of lipid-soluble matter; insoluble in petroleum ether) | |

^a These analyses were carried out by Mr. E. F. Corcoran under the direction of one of the authors (D. L. F.).

the solutes were transferred into petroleum ether by the addition of water. The extracts were then washed once with water, dried over sodium sulfate, concentrated *in vacuo* to 1 l. and kept over a layer of 20% methanolic KOH for a night. After the elimination of the alkali by washings, the dried, orange-brown solution was concentrated to 150 ml. and developed on a calcium hydroxide-celite column (3:1; 28 × 7.5 cm.) with petroleum ether containing 2% acetone. In the following chromatogram the figures denote the height of zones, in millimeters:

2 two red streaks
 2 light pink: fluorescing compound A
 11 several minor pigment zones
 17 empty interzone
 10 pale orange
 10 empty interzone
 13 orange: β -carotene (relatively main pigment)
 2 colorless (overlapping with pigments): fluorescing compound B
 10 very pale orange
 10 almost colorless: fluorescing compound C
 50 pale yellow
 115 colorless
 Filtrate: fluorescing compound D.

By systematic fractionation and repeated re-chromatographing which will not be described in detail, the compounds A, B, C, and D were

obtained in almost colorless, and chromatographically homogeneous, solutions. Figure 1 is based on light absorption measurements taken on a Beckman spectrophotometer.

SUMMARY

A deep-sea mud sample (age up to 960 years) was found to be free of phytofluene or other colorless polyenes, although epiphasic carotenoid pigments such as β -carotene [cf. (3)] were present. The observed fluorescence of the extracts was caused mainly by four non-polyenic compounds (Fig. 1).

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β -Glucuronidase

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INTRODUCTION

In view of the important role of glucuronic acid conjugation in detoxication and in steroid metabolism, the widespread occurrence in animals, plants, and bacteria of an enzyme specific for the hydrolysis of β -glucuronides is of considerable interest. The existence of this enzyme was first demonstrated by Masamune (1) who developed a method for its purification from ox kidney. In 1936 Oshima (2) carried the purification a step further. Since this time many articles about β -glucuronidase have appeared. Fishman (3) achieved a 140-fold purification of β -glucuronidase from ox spleen. Graham (4) claimed to have concentrated the enzyme 315-fold, starting with acetone-precipitated ox spleen. According to him, this was possible because of a two-fold increase in activity brought about by incubation of the water extract for 6 hr. at 25–30°C., followed by dialysis overnight. However, Mills (5) could not repeat this, neither could we.

OCCURRENCE OF β -GLUCURONIDASE

As is indicated in Table I the activities of β -glucuronidase vary considerably in different animal species. The low values for this enzyme in the pig are startling. It has been stated by several authors that the spleen is the organ richest in β -glucuronidase, but we do not find this to be true.

METHOD OF ESTIMATION

We have estimated glucuronidase activity by the method of Talalay, Fishman, and Huggins (6) with certain modifications as described below:

Using a serological pipet place 0.1 ml. of the properly diluted enzyme in a 15 \times 1.8 cm. test tube. Wash this down with 1.0 ml. of 0.1 M pH 4.5 acetate buffer. Place the tube in a thermostat bath at 38°C. and allow it to reach that temperature. Now add

1 ml. of 0.043% phenolphthalein glucuronide,¹ which is at 38°C., and mix. Allow to digest anywhere from 1 min. to 4 hr. or more, depending upon the activity of the glucuronidase. Stop enzyme action by adding from a pipet 10 ml. of 0.5 *M* NaOH-glycine buffer of pH 10.5. Read in a colorimeter, using a green filter. Plot the readings against minutes and interpolate to find out how many minutes digestion were required to give a reading of 100. Dividing 100 by this number of minutes will give the units of β -glucuronidase in 0.1 ml. of enzyme solution. We have standardized our colorimeter, using known amounts of phenolphthalein in alcohol and water. It is to be noted that our colorimetric reading of 100, using a green filter was found to be equivalent to 0.032 mg. of phenolphthalein and that this value will vary with each colorimeter used. That quantity of β -glucuronidase which will give a reading of 100 in 1 min. contains 100 units; that quantity which will give a reading of 100 in 10 min. contains 10 units.

TABLE I
 β -Glucuronidase Units
(Per g. of fresh tissue)

| | Liver | Kidney | Spleen |
|-------------|-------|--------|--------|
| Ox | 650 | — | — |
| | 315 | — | 65 |
| | 310 | 125 | 80 |
| Pig | 1.5 | 2.9 | 4.3 |
| White rat | 625 | 240 | 512 |
| | 670 | 256 | 546 |
| Guinea pig | 185 | 35 | 230 |
| Hen | 216 | 305 | 77.5 |
| Chicken | 195 | 255 | 100 |
| Turkey | 305 | 203 | 330 |
| Brook trout | 470 | 450 | 180 |

When crude preparations of β -glucuronidase were employed we added the 10 ml. of NaOH-glycine buffer and then centrifuged the solution until clear. We have also run blanks, the values for which were subtracted from the test. However, with highly colored material, such as blood, this procedure cannot be employed and the original method of precipitation of impurities used by Talalay *et al.* (6) must be followed.

PURIFICATION

Beef liver was chosen as our starting material since this contains a rather high concentration of β -glucuronidase, since one of us has already

¹ This reagent can be obtained from the Sigma Chemical Company, St. Louis, Missouri.

worked for many years with beef liver, and since this material can be readily obtained from slaughter houses, or from stores. Our procedure, the first steps of which are identical with those employed for the preparation of crystalline catalase (7), is as follows:

Put 6 lb. of fresh beef liver through a meat grinder 4 times. To 300-g. portions add 400-ml. portions of 35% dioxane. This dioxane may contain acid, in which case it should be recrystallized twice before use.

The material is allowed to filter by gravity at room temperature overnight. Evaporation is prevented by covering the filtration funnels with watch glasses. The next day one adds 20 ml. of dioxane to every 100 ml. of filtrate, cools for 8-10 hr. in the ice chest, and filters in the ice chest. The following day one adds 10.2 ml. of dioxane to every 100 ml. of the filtrate, cools 8-10 hr., and filters in the ice chest. The material which is filtered off can be used for the preparation of crystalline catalase.

The filtrate is cooled to about -14°C ., and to every 100 ml. one adds 10 ml. of dioxane. The precipitate is immediately centrifuged off at a low temperature and dissolved in about 120 ml. of water. The glucuronidase is now adsorbed by stirring with 400 ml. of 1.8% of $\text{Ca}_3(\text{PO}_4)_2$ suspension at pH 5.7. The adsorption complex is centrifuged down and the supernatant liquid, which will be entirely free of enzyme, is discarded. The enzyme is eluted by stirring with 20 ml. 0.7 *M* pH 7.4 phosphate buffer and about 250 ml. of water, followed by centrifuging. This elution is repeated twice more. The three eluates are combined. This solution should contain about 75,000 units of β -glucuronidase.

The glucuronidase is now salted out by adding an equal volume of neutral, saturated ammonium sulfate. The precipitate is filtered by gravity in the ice chest and is kept from drying out by the use of watch glasses. The precipitate is dissolved in about 50 ml. of water and again salted out by adding an equal volume of neutral, saturated ammonium sulfate. It is now centrifuged down hard in 50-ml. centrifuge tubes. The supernatant is discarded and the precipitate is mixed with a very little water and dialyzed in the ice chest against many changes of distilled water. At this point the volume should not be more than about 25 ml. After 2 or 3 days of dialysis the greater part of the β -glucuronidase will be present in the precipitate. This is centrifuged off and washed twice with cold distilled water. The enzyme is now extracted by stirring the precipitate with about 5 ml. of 1 *M* acetate buffer of pH 4.5 at 38°C . for 20 min., followed by centrifuging. The faintly yellow extract should contain about 20,000 units of enzyme. It is next dialyzed in the ice chest against several changes of distilled water. A portion of the β -glucuronidase will precipitate as tiny spheroids. These, when suspended in water, will possess an activity of about 1800 units/mg. of dry weight. This indicates a concentration of about 6000-fold, starting with fresh liver containing 315 units/g.

PROPERTIES OF β -GLUCURONIDASE

Heat Stability

β -Glucuronidase has been found to be fairly stable to heat; it does not lose its activity on heating for 30 min. at 50°C . It begins to lose

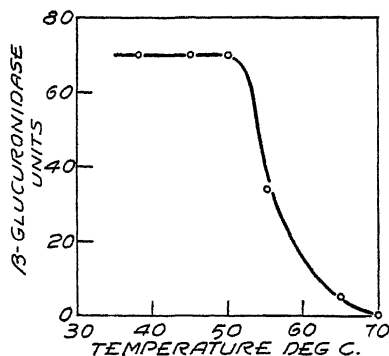


FIG. 1. Effect of heating upon activity.

activity slowly above 50°C. and then very rapidly above 55°C. The change of activity on heating for 30-min. intervals at different temperatures is shown in Fig. 1.

Effect of pH

Mills (5) has found that the pH-activity curve for the hydrolysis of different conjugated glucuronides by β -glucuronidase from ox spleen shows two peaks at two slightly different pH values, thereby indicating the presence of two different enzymes. Using 1-mentholglucuronide he found the pH optima to be at 4.5 and 5.0; for phenylglucuronide at 4.5 and 5.2; and for phenolphthalein glucuronide at 4.5 and 5.2. The existence of two peaks in the pH-activity curves was obtained with mouse

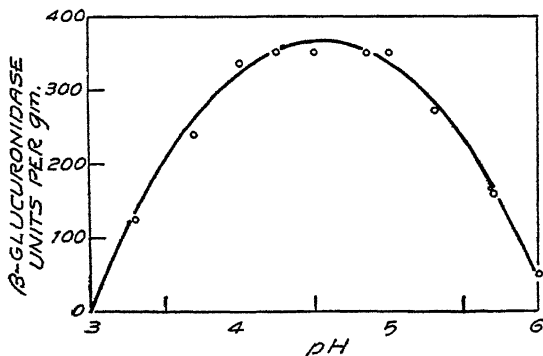


FIG. 2. pH Activity curve of crude enzyme.

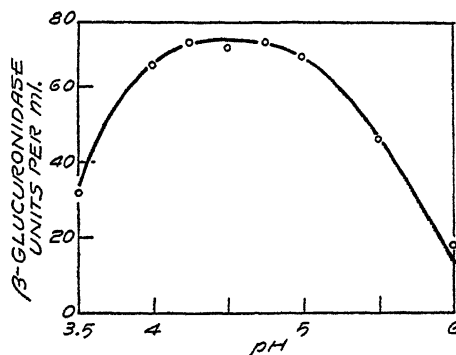


FIG. 3. pH Activity curve of purified enzyme.

liver, spleen, and kidney (8,9), and the same was true also with ox spleen.

Talalay, Fishman, and Huggins (6) reported that using phenolphthalein glucuronide as substrate and crude preparations of β -glucuronidase from pooled liver, spleen, and kidney of the mouse, they obtained only one pH optimum, which was at 4.5. We, too, failed to demonstrate the existence of two different β -glucuronidases in liver or spleen, since our results show only one pH optimum. Figures 2 and 3 show the pH-activity curve of crude liver and of the purified enzyme, respectively. The similarity of the two curves indicates that β -glucuronidase is

TABLE II
Inhibition of β -Glucuronidase (50 Units)

| Inhibitor | Quantity employed mg. | Per cent of inhibition |
|--------------------|--------------------------|---------------------------|
| Sodium fluoride | 0.1-2.0 | 0 |
| Cobaltous chloride | 0.1-2.0 | 0 |
| Manganous chloride | 0.1-2.0 | 0 |
| Magnesium chloride | 0.1-2.0 | 0 |
| Potassium cyanide | 0.1-2.0 | 0 |
| Sodium azide | 0.1-2.0 | 0 |
| Silver nitrate | 0.1 | 100 |
| Silver nitrate | 0.05 | 80 |
| Cupric sulfate | 5.0 | 100 |
| Cupric sulfate | 2.0 | 60 |
| Cupric sulfate | 1.0 | 45 |
| Cupric sulfate | 0.1 | 20 |
| Uranium nitrate | 2.0 | 50 |
| Uranium nitrate | 0.2 | 30 |

equally affected by the hydrogen-ion concentration, whether in crude or in purified form. The figures indicate further that the enzyme is stable over a wide range of pH.

Inhibitory Effect of Metallic Ions and Other Substances

It will be seen from Table II that β -glucuronidase is fairly stable toward certain metallic ions. Of all the reagents listed in Table II, only silver ions caused complete inhibition at a low concentration. Copper ions also inhibited but only when used at a relatively high concentration. Uranium ions behaved similarly.

Crystalline trypsin did not inactivate β -glucuronidase.

Enzyme Concentration Effect

As shown in Fig. 4, a straight line is obtained when enzyme activity is plotted against time of digestion at 38°C., indicating a reaction of zero order.

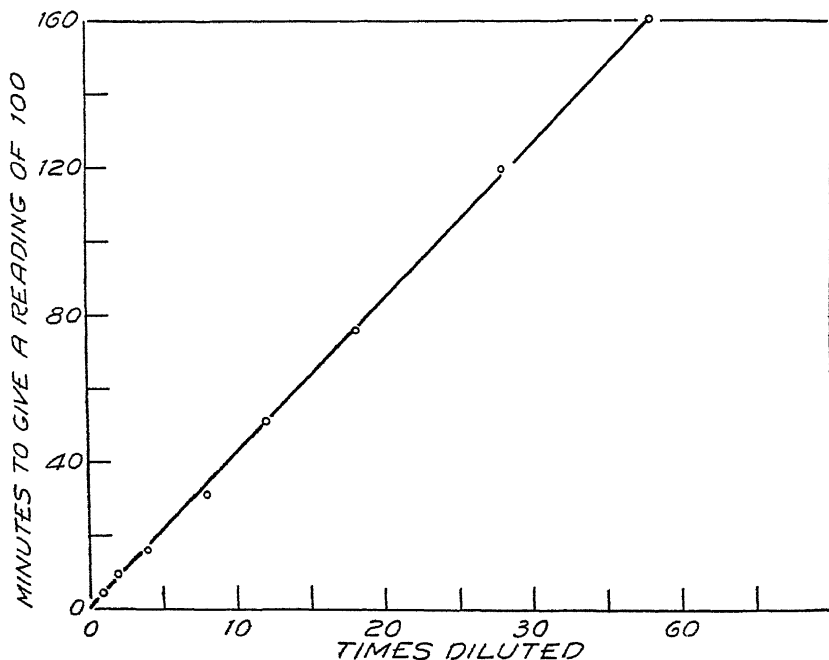


FIG. 4 Enzyme activity as related to digestion time.

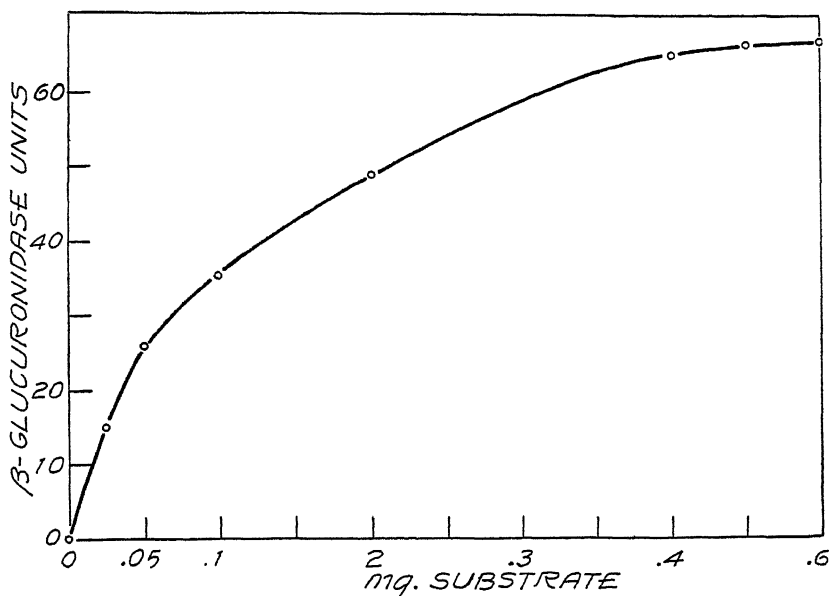


FIG. 5. Substrate concentration effect.

Substrate Concentration Effect

Figure 5 shows that with increasing substrate concentration, enzyme activity increases until it reaches a maximum at a substrate concentration of 0.6 mg. of phenolphthalein glucuronide (in the 2.1 ml. of digest).

Effect of Temperature

As is shown in Table III, β -glucuronidase has normal temperature coefficients.

Chemical Nature

Highly purified β -glucuronidase gives the usual protein tests but no ninhydrin test. It appears to be a globulin.

TABLE III

| Temperature interval °C. | Temperature coefficient |
|-----------------------------|-------------------------|
| 20-30 | 2.6 |
| 30-40 | 1.7 |
| 40-50 | 1.4 |
| 50-60 | 0.65 |

ACKNOWLEDGMENT

We wish to express our gratitude to the Rockefeller Foundation for financial assistance.

We wish to thank Professor S. L. Leonard of this university for a supply of phenolphthalein glucuronide.

SUMMARY

Figures are given for the activities of β -glucuronidase in liver, kidney, and spleen of several animal species.

A modification is described of the method of Talalay, Fishman, and Huggins for estimating β -glucuronidase activity. A procedure is described whereby the β -glucuronidase in beef liver can be concentrated about 6000-fold. Some of the properties of this enzyme are described.

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LETTERS TO THE EDITORS

Identification of the Sugars in Crystalline Tomatin

In an earlier paper (1) it was stated that tomatin (mol. wt. 1033) is a glycosidal alkaloid, isolated from red currant (*Lycopersicon pimpinellifolium*) tomato leaves, and exhibits antifungal activity. It is composed of an aglycone fraction, tomatidine (mol. wt. 415), and a tetrasaccharide moiety. We have now determined that the carbohydrate fraction consists of one molecule each of xylose and galactose, and two molecules of glucose.

It is of interest to note that Kuhn and Löw (2) have isolated a glycosidal alkaloid, demissine, from the leaves of *Solanum demissum*. Their compound consists of an aglycone portion, dihydrosolanidine, and one molecule each of xylose and galactose, and two molecules of glucose. We have indicated in an earlier paper (1) that tomatin and tomatidine are not identical with solanine and solanidine. It is likewise true that the former two compounds are not the same as demissine and dihydrosolanidine, although the tetrasaccharide moiety is the same; however, at the present time, we do not know whether they are attached to the aglycone portion in the same sequence. A further characterization of the tomatidine portion of tomatin will be reported elsewhere.

One gram of crystalline tomatin was hydrolyzed by refluxing for 1 hr. in 20 ml. of 1.0 *N* hydrochloric acid solution. As hydrolysis proceeded, a crystalline aglycone hydrochloride, tomatidine hydrochloride, precipitated from the hot solution. At the end of 1 hr. boiling, the solution was cooled and the precipitate collected in a fritted glass funnel, washed with distilled water and dried in a vacuum oven at 80°C. The yield of unpurified tomatidine hydrochloride was 433 mg., or equivalent to 398 mg. of tomatidine. The total reducing sugars in the filtrate, determined by the titration method of Schoorl (3), amounted to 576 mg. (calculated as glucose).

The remaining filtrate (97 ml.) was neutralized to pH 7.2 by passing it through an anion exchange column of Amberlite IR-4B (75 g.), and the column was washed with distilled water. In addition to removing the hydrochloric acid, the small amount of tomatidine hydrochloride in solution was also removed. The neutralized sugar solution (407 ml.) was concentrated to 10 ml. under reduced pressure at 40–50°C. This concentrate was then used for the qualitative and quantitative determination of the sugars present in the tomatin hydrolysate.

The qualitative identification of the sugars in the tomatin hydrolysate was accomplished with the aid of the ascending paper chromatographic technique developed in this laboratory (4). It was determined that only three sugars were present—glucose, galactose, and xylose. The sugars were identified by their specific position on the paper chromatogram and by their color development when the chromatogram was sprayed with either naphthoresorcinol, α -naphthol, phloroglucinol, orcinol, or ammoniacal

silver nitrate solutions and then heated in an oven. In this ascending chromatographic method, xylose was completely separated from glucose and galactose when a solvent consisting of *n*-butanol, 35 parts, ethanol, 52 parts, and water, 13 parts, was used.

For the quantitative determination of the sugars present, the concentrated neutralized hydrolysate was streaked across the lower edge of No. 4 Whatman filter papers, 23 × 28 cm. Since only approximately 1 ml. of the concentrate could be placed on each sheet, it was necessary to set up 10 such sheets. The chromatograms were developed by the *n*-butanol-ethanol-water solvent for 24 hr., then removed, and dried in air. A very thin strip was cut from each sheet and sprayed with ammoniacal silver nitrate to locate the exact position of the sugars on each sheet. Having located the position of the sugars, the chromatograms were cut in such a way as to remove the xylose band from the glucose and galactose bands, the latter two of which did not separate completely from each other. The paper strips containing xylose and those containing glucose plus galactose were leached separately with a total of 250 ml. of distilled water. Aliquots of these solutions were analyzed for total reducing sugars by the titration method of Schoorl (3). From model experiments it was determined that 95–100% recovery of xylose and 96–100% recovery of glucose plus galactose were obtained in this manner. Since a portion of the xylose was converted to furfural during the initial acid hydrolysis step, it was necessary to make a correction for such a loss; on this basis only one molecule of xylose was found.

The solution containing glucose plus galactose was frozen and dried by lyophilization. The glucose plus galactose fraction, with suitable, known control mixtures, were then fermented, according to the method reported by Porter and Fenske (5), using *Saccharomyces bayanus*, NRRL No. Y966, to remove the glucose. Since this particular yeast is specific for fermenting glucose, the amount of sugar remaining after 48 hr. was considered to be galactose, and glucose was determined by difference. A ratio of 1:1.97 for galactose to glucose was found. A control solution, originally containing 200 mg. of glucose and 100 mg. of galactose, fermented under identical conditions gave a titration value equivalent to 102 mg. of galactose. From the results obtained in this investigation it is concluded that the tomatin molecule consists of an aglycone portion, tomatidine, and one molecule each of xylose and galactose, and two molecules of glucose.

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Protogen and Acetate in *Tetrahymena*¹

Protogen, a required growth factor for the animal microorganism *Tetrahymena* (1), has been identified (2) with the acetate factor of Guirard, Snell, and Williams (3) and the pyruvate oxidation factor of O'Kane and Gunsalus (4) for lactic acid bacteria. It appears, therefore, that protogen controls reactions from pyruvate to acetate, and the latter can replace protogen.

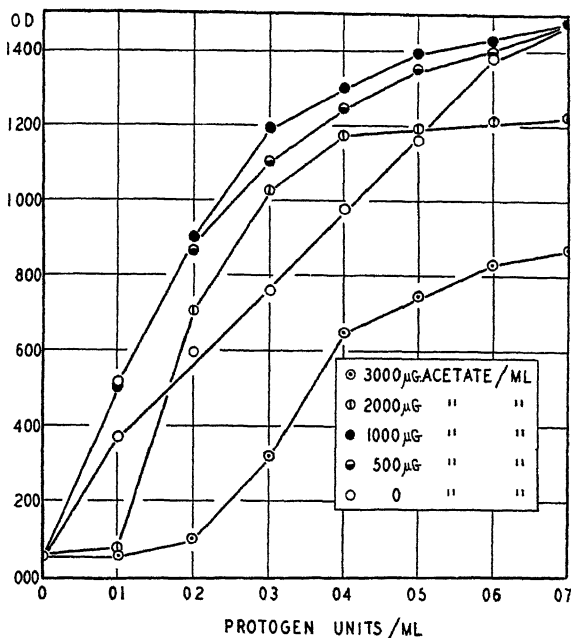


FIG. 1. Dose response of *Tetrahymena geleii* W to protogen in medium 2C (6), and in same medium plus varying concentrations of acetate (as the sodium salt).²

It has been shown (5) that the growth of *Tetrahymena* fails in the absence of protogen even when acetate is present. Quantitative studies demonstrate, however, that acetate is active in sparing protogen. While high concentrations of acetate (above 3 mg./ml) are inhibitory, the addition of 1 mg. acetate/ml. of medium reduces the protogen requirement by approximately one half for half-maximum yield (see Fig. 1).

These results suggest that one of the functions of protogen in this animal organism is concerned with the production of acetate, and it may be that the metabolic path-

¹ Aided by a grant from the Research Corporation and a grant recommended by the Committee on Growth acting for the American Cancer Society.

² The protogen was kindly supplied by Dr. E. L. R. Stokstad.

ways are the same as those operating in the lactic acid bacteria. In addition to this function, protogen must control one or more other reactions in *Tetrahymena*.

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Reproduction of Mice Kept on Rations Low in Vitamin B₁₂

Reproduction failures of rats and mice on synthetic or whole-plant rations have been described and attributed to a lack of animal protein factor (APF) or vitamin B₁₂ (1-4). We have succeeded in breeding mice, kept on whole-plant rations, for several generations; nearly normal reproduction and mortality rates were observed although the animals showed symptoms of vitamin B₁₂ deficiency.

The experimental diets contained 22-4% of crude protein ($N \times 6.25$). Diet III had the following composition: expelled sesame-oil cake-meal, 46.5%; ground yellow corn, 46.5%; cottonseed oil containing 0.2% of percomorphum and 0.2% of wheat germ oil, 5%; U.S.P. salt containing 0.1% of $CoCl_2$, 2%; 10 B-complex vitamins as described (4). Albino mice were kept on a commercial stock ration, containing 24% of crude protein (13.5% of animal origin) prior to the start of the experiments. Pregnant females were put in individual screen-bottomed cages and given diet III. Litters were

TABLE I
Reproduction of Mice Kept on Different Diets

| Diet | No. of litters | No. of litters weaned | Weight of young at 4 weeks | Weight change of mothers during lactation | No. of young dead between 2nd and 4th week |
|---------------------|----------------|-----------------------|----------------------------|---|--|
| III, 1st generation | 6 | 6 | 15.2 | 0 | 2 |
| III, 2nd generation | 10 | 9 | 9.2 | 0 | 5 |
| III, 3rd generation | 12 | 10 | 9.2 | 0.7 | 11 |
| III, 5th generation | 13 | 13 | 11.5 | 0 | 6 |
| I, 2nd generation | 9 | 8 | 10.3 | -0.5 | 4 |
| Control | 10 | 10 | 15.4 | 0.5 | 4 |

TABLE II
*Growth Response of Mice Raised on Diet III to Single
 Injections with Crystalline Vitamin B₁₂*

| No. of mice | Dose of vit. B ₁₂ | Wt. gain/day/animal, 2 weeks before injection | Wt. gain/day/animal, weeks after injection |
|-------------|------------------------------|--|---|
| | $\mu\text{g.}$ | g. | g. |
| 18 | 0.01 | 0.24 | 0.30 |
| 25 | 0.5 | 0.21 | 0.63 |

reduced to seven. The mothers were killed 7 weeks after the birth of the litters and these were kept together on diet III until females became pregnant; the mice were then separated and the same operation repeated. In the course of 18 months, five generations of mice were obtained by brother-sister breeding.

In a previous study, a very poor reproduction in rats and mice on a soybean-corn ration had been observed (4). Therefore, pregnant female mice, raised on diet III and born from mothers raised on diet III, were put on this soybean-corn ration (diet I) which differed from diet III in that sesame meal was replaced by commercial solvent-extracted soybean meal. The animals and their litters were treated as described and 3 generations were raised on this diet.

The young of the experimental groups grew more slowly than the controls but the mortality was nearly the same in both groups (Table I). Single intraperitoneal injections of 0.5 $\mu\text{g.}$ /animal of crystalline vitamin B₁₂ (Merck) resulted in growth improvement for a period of 2 weeks, while 0.01 $\mu\text{g.}$ was without effect (Table I). Kidney hypertrophy has been observed in rats deficient in vitamin B₁₂ (5,6); the ratio between body weight and weight of the kidneys in mice raised on diet III in the 3rd and 4th generation was measured and found to be 50.1 ± 5.2^1 in 55 adults weighing 20-32 g., while in 35 controls weighing 22-32 g. this ratio was 55.5 ± 6.7^1 (For difference between means, $p < 0.01$.)

Diets I and III were analyzed with *Lactobacillus leichmannii* and found to possess 0.4 and 0.5 $\mu\text{g.}/100 \text{ g.}$ of vitamin B₁₂ activity, while with an assay procedure using *Lactobacillus lactis* Dornier, 0.7 and 0.4 $\mu\text{g.}/100 \text{ g.}$, respectively, were found.²

These results indicate that mice were able to reproduce on diets low in vitamin B₁₂, according to microbiological assay, and insufficient to cause normal growth and to prevent kidney hypertrophy.

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¹ Standard error.

² The *L. leichmannii* assay was made by the Wisconsin Alumni Research Foundation, the *L. lactis* Dornier assay by Merck and Co., Research and Development Division. We are deeply indebted to Dr. H. B. Woodruff for this assay.

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The Effect of B₁₂ Concentrates on the Reduction of S—S Groups¹

The demonstration that vitamin B₁₂ concentrates have a methyl-sparing action in rats (1) led us to a study of the influence of this vitamin on transmethylation reactions *in vitro*. It was found that "Rubramin,"² a B₁₂ concentrate, increases the reduction of homocysteine and decreases the net oxidation of homocysteine in rat liver slices (Table I). Since homocysteine appears to be the direct acceptor of methyl (2,3), a greater synthesis of methionine may be expected in the presence of B₁₂ under conditions where the reduction of homocysteine is limiting. This has been found to be so. The degree of reduction of glutathione is similarly affected by B₁₂ concentrates. Crystalline B₁₂ and B_{12a} have little or no activity under these conditions, and not all samples of "Rubramin" are active. Nevertheless, it seems probable that B₁₂, perhaps in a hitherto undescribed form, is the active factor in these concentrates. This is suggested by the more striking *in vitro* effect of "Rubramin" in liver slices from B₁₂-deficient rats than in the normal rats, and the fact that activity has always been associated with the B₁₂ color after paper chromatography with various solvents. Preliminary experiments with these fractions have demonstrated the presence of microbiologically-active B₁₂ components which do not correspond in *R_f* values or in absorption spectra to either crystalline B₁₂ or B_{12a}.

These results suggest a B₁₂ component acting as a hydrogen carrier to —S—S— groups. This may explain the effect of B₁₂ on methionine formation and the fact that B₁₂ may be at least partially replaced as a microbiological growth factor by reducing agents (5,6) or by lowering the oxygen tension of the medium (7,8). Other apparently diverse *in vivo* activities of B₁₂ which have been reported may be the result of the influence of B₁₂ on glutathione and —SH groups, which in turn can activate a wide variety of enzyme systems (9).

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¹ Aided by a grant from the U. S. Public Health Service.

² "Rubramin" is the B₁₂ concentrate prepared by E. R. Squibb & Sons.

The Effect of B₁₂ Concentrates on the Reduction of S-S Compounds

| | Oxidized glutathione | Reduced glutathione | Homocystine | Homocysteine | "Rubramin" | SH in mg.—% | | |
|----|----------------------|---------------------|-------------|--------------|------------|-------------|-------------|---|
| | | | | | | Total | Less tissue | Net reduction due to addition of "Rubramin" |
| 1 | | | | | | 1.5 | | |
| 2 | + | | | | | 1.8 | 0.3 | |
| 3 | + | | | | + | 4.3 | 2.8 | +1.0 |
| 4 | | + | | | | 6.8 | 5.3 | |
| 5 | | + | | | + | 11.2 | 9.7 | +2.4 |
| 6 | | | | | + | 3.0 | 1.5 | |
| 7 | | | | | | 1.0 | | |
| 8 | | | + | | | 1.1 | 0.1 | |
| 9 | | | + | | + | 2.0 | 1.0 | +0.6 |
| 10 | | | | + | | 5.6 | 4.6 | |
| 11 | | | | + | + | 9.7 | 8.7 | +3.8 |
| 12 | | | | | + | 1.8 | 0.3 | |

Figures are average of duplicates ± 0.1 mg.—%. 5 μ g. "Rubramin," 250- μ g. substrate, as indicated with 2 mg. glucose and 10 mg. dry wt. of slices in 2 ml. Krebs phosphate buffer. Incubated 3 hr. in air. Mercapto group determined by modification of Lugg's method (4). There is no B₁₂ effect in boiled tissue. In the absence of tissue, —SH compounds are almost completely oxidized under these conditions. B₁₂ catalyzes this autooxidation.

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The Requirements of Rats for Methyl Groups and Vitamin B₁₂ in the Production of Leucocytes¹

We have previously suggested that folic acid functions in the choline oxidase enzyme system (1), and Dubnoff has demonstrated that this enzyme is essential for labilizing the methyl groups of choline (2). We have studied the requirement of labile methyl

¹ Research paper No. 897, Journal Series, University of Arkansas. This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

TABLE I

The Effects of Betaine and Vitamin B₁₂ on Peripheral Blood Leucocytes of Rats

| Diet | No. of rats | Peripheral leucocytes (thousand/ μ l.) | | | |
|-------------------------|-------------|--|-----------------------------------|------------------|-------------------------|
| | | Initial ^a | Betaine supplemented ^b | Change | |
| Basal | 3 | 8.43 | 7.47 | average -0.96 | range +0.10 to -2.60 |
| Basal + B ₁₂ | 8 | 6.81 | 11.91 | +5.10 | +2.70 to +8.50 |
| Basal + 0.6% methionine | 8 | 19.10 | — | — | — |

^a Counts taken on 65th experimental day.^b Nine days after addition of 0.084% betaine.

for leucocyte formation and also the effect of vitamin B₁₂ on the utilization of methyl groups. Weanling Sprague-Dawley rats were given a basal diet of isolated soybean protein,² 18 g.; sucrose, 67 g.; Crisco, 8.0 g.; salt mix (3), 4 g.; cod liver oil, 2 g.; choline chloride, 0.1 g.; inositol, 10 mg.; thiamine chloride, 1.5 mg.; riboflavin, 5.0 mg.; nicotinamide, 2.0 mg.; calcium pantothenate, 1.0 mg.; pyridoxine hydrochloride, 0.5 mg.; menadione, 0.025 mg.; biotin, 0.005 mg., and folic acid, 0.5 mg. This diet contains approximately 0.27% methionine, supplied by the soybean protein. One group of eight rats received the unsupplemented basal diet and another group of eight rats were given this diet supplemented with 5.0 μ g. of vitamin B₁₂³/100 g. of diet. The rats were given these diets for 65 days; during this period the average total weight gain was 6.2 g./rat for the group receiving the basal diet and 15.1 g./rat for those receiving the diet supplemented with vitamin B₁₂. Five of the rats receiving the basal diet died during the 65-day period; there were no deaths in the group receiving B₁₂. On the 65th experimental day complete blood counts were made and 0.084% betaine was added to both diets. After 9 days on the modified diets, complete blood counts were again taken. There were no significant differences in total red cell count, hemoglobin, or hematocrit. The peripheral leucocyte counts are shown in Table I. Rats in both groups were leucopenic on the 65th experimental day; in contrast, rats receiving the basal diet supplemented with 0.6% methionine but no B₁₂ had an average of 19,100 leucocytes/ μ l. of blood. The addition of betaine to the basal diet did not increase the leucocyte counts while the addition of betaine to the B₁₂ diet resulted in an increased leucocyte count for all the rats. The data suggest that the low-methionine and low-choline contents of the basal diet were inadequate to supply sufficient methyl groups for leucocyte formation. They show that methionine in the absence of vitamin B₁₂, or betaine in the presence of vitamin B₁₂, can function in the production of leucocytes. The results suggest, therefore, that vitamin B₁₂ may be essential for the utilization of the methyl groups of betaine.

² "Alpha protein," from the Glidden Company, Chicago, Illinois.³ The vitamin-B₁₂ concentrate (Rubramin) used in this study was kindly supplied by E. R. Squibb and Sons, New York, New York.

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Effect of Aureomycin on the Growth of Weaned Pigs¹

Cunha (1), using a corn-peanut meal diet, demonstrated a difference in the response of growing swine to vitamin-B₁₂ concentrates, and concluded that the concentrate prepared by Lederle Laboratories from a *Streptomyces aureofaciens* fermentation contained vitamin B₁₂ plus some other factor. Other workers (2) have made similar observations with chicks.

The experimental animals in these tests were weaned Chester White pigs from the Hormel Foundation herd. In general, pigs from this herd are unthrifty and exhibit an enteritis-type diarrhea soon after weaning (3). The basal diet had the following composition: corn 41, oats 20, wheat middlings 10, alfalfa meal dehydrated 10, soybean oil meal 7, tankage (60%) 10, commercial mineral 2, and 0.25 lb. irradiated yeast (9000 U.S.P. units of vitamin D/g.) per ton. Eight pigs were used per lot. In Expt. 1 the pigs were confined in 6 × 15 ft. pens with concrete floors in a temperature-controlled farrowing barn. In the second experiment, each lot of 8 pigs was confined to an 8 × 8 ft. pen with a concrete floor in a partially heated farrowing barn. Feed and fresh water were available at all times. The pigs were weighed weekly during the 4-week experimental periods.

The data for Expt. 1 show that the average daily gains made by pigs on the basal diet could be increased by supplementation with animal protein factor (APF) concentrate No. 3, a material which contains 12.5 mg. of vitamin B₁₂ activity/lb. However, the rate of gain was increased considerably more when the diet was supplemented with the same vitamin-B₁₂ concentrate plus aureomycin. This latter combination gave the same growth response as the APF concentrate No. 1 which contained 4.2 mg. of aureomycin/g. (4). Pigs in lots 1 and 3 exhibited more diarrhea than pigs in lots 2 and 4. The animals in lots 2 and 4 showed more smoothness and general thriftiness than the control pigs, and during the last week of the experiment gained an average of almost 1 lb./day. The feed efficiency was increased from 6.4 to 3.1 lb. of feed/lb. gain by the addition of dietary supplements containing aureomycin; vitamin-B₁₂ supplementation also increased feed efficiency.

In Expt. 2, 1 mg. of vitamin B₁₂ as concentrate No. 3 improved the basal diet only slightly. Aureomycin alone also improved the quality of the diet, and when fed at a level of 1.25 g./100 lb. of feed, gave the same growth response as the APF concentrate No. 1. Pigs fed diets containing aureomycin or the APF concentrate containing aureo-

¹ Hormel Institute publication No. 50.

mycin did not have diarrhea. The feed efficiency was again increased by the addition of supplements containing vitamin B₁₂ or aureomycin.

These data indicate that unthrifty pigs fed a diet of natural feedstuffs including animal byproducts may respond to additional dietary vitamin B₁₂. This apparent deficiency condition can be overcome by feeding, under certain environmental conditions, a crude concentrate of vitamin B₁₂ and aureomycin or pure aureomycin alone. Therefore "disease level" or intestinal flora have an important bearing on dietary requirements of vitamin B₁₂.

TABLE I

Effect of APF Concentrates and Aureomycin on the Growth of Weaned Pigs

| Expt. no. | Lot no. | Diet supplement, 100 lb. feed | Number of pigs | | Average starting weight | Average daily gain | Feed/gain |
|-----------|---------|---|----------------|----------------|-------------------------|--------------------|----------------|
| | | | Start | End | | | |
| 1 | 1 | None..... | 8 | 8 | lb. 22 | lb. 0.23 | lb./lb. 6.4 |
| | 2 | APF No. 1, ^a 0.5 lb..... | 8 | 8 | 22 | 0.70 | 3.1 |
| | 3 | APF No. 3 ^b 0.2 lb..... | 8 | 8 | 22 | 0.46 | 3.6 |
| | 4 | APF No. 3, 0.2 lb., + 1.25 g. aureomycin ^c | 8 | 8 | 23 | 0.74 | 3.1 |
| | . | | | | | | |
| 2 | 1 | None..... | 8 | 6 ^d | 30 | 0.46 | 5.6 |
| | 2 | APF No. 1, 0.5 lb..... | 8 | 8 | 27 | 0.86 | 3.0 |
| | 3 | APF No. 3, 0.081 lb..... | 8 | 8 | 29 | 0.51 | 4.2 |
| | 4 | Aureomycin, 0.5 g..... | 8 | 8 | 29 | 0.67 | 3.7 |
| | 5 | Aureomycin, 1.25 g..... | 8 | 8 | 28 | 0.88 | 2.9 |
| | 6 | Aureomycin, 0.5 g. + 0.081 lb. APF No. 3..... | 8 | 8 | 27 | 0.76 | 3.5 |
| | 7 | Aureomycin, 1.25 g. + 0.081 lb. APF No. 3..... | 8 | 7 ^e | 28 | 0.86 | 3.2 |

^a Lederle APF No. 5, Lot 24. Kindly supplied by Lederle Laboratories, Pearl River, N. Y.

^b Merck concentrate No. 3, containing 12.5 mg. vitamin B₁₂/lb. Kindly supplied by Merck & Co., Rahway, New Jersey.

^c Aureomycin hydrochloride, supplied by Lederle Laboratories.

^d One pig died; one developed bloody diarrhea.

^e One pig removed because it did not eat.

Aureomycin at a level of 1.25 g./100 lb. of feed gave the same growth stimulation as an equivalent amount of Lederle APF concentrate, or a combination of vitamin B₁₂ (Merck No. 3) and pure aureomycin. These data suggest that the plus factor in APF concentrates is an antibiotic.

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Relationship of the *Lactobacillus bulgaricus* Factor to Pantothenic Acid

The existence of an unidentified growth factor required by *Lactobacillus bulgaricus* (Sarles' strain) and certain other lactic acid bacteria has been reported recently (1). Modifications in the vitamin content of the medium used for assay led to the observation that large amounts of pantothenate (relative to the amounts needed by other lactic acid bacteria, e.g., *L. arabinosus* 17-5) produce maximum growth in the basal medium without the addition of the *L. bulgaricus* factor (LBF). Table I shows the amounts of yeast extract (as a source of LBF) and pantothenate required for growth. Essentially the same interchangeability of LBF and pantothenate exists for two other organisms, *L. helveticus* 80 and *L. bulgaricus* 10 (A.T.C.C. No. 8018) (2), earlier demonstrated to require LBF (3).

Figure 1 illustrates the effect of varying levels of pantothenate on the response of *L. bulgaricus* to a purified LBF concentrate which has a potency of 1 unit/ μ g. of solids. A unit is defined as that amount of LBF in 1 mg. of Basamin Busch yeast extract. In the complete absence of pantothenate, growth occurs; but maximum growth is not attained over the range of LBF studied. The 5- μ g. level is the amount present in the medium used for assay of LBF. Even when pantothenate is in excess

TABLE I

Relative Amounts of a Source of LBF and Calcium Pantothenate Needed to Give Growth of *Lactobacillus bulgaricus*

| Yeast extract ^a | | Calcium pantothenate | |
|----------------------------|------------------------|----------------------|------------------------|
| mg./10 ml. | Turbidity ^b | μ g./10 ml. | Turbidity ^b |
| 0 | 91 | 0 | 92 |
| 0.45 | 79 | 5 | 92 |
| 0.9 | 49 | 9 | 86 |
| 1.2 | 39 | 13 | 37 |
| 1.5 | 38 | 15 | 33 |

^a The basal medium used to obtain the response to yeast extract contains 5 μ g. calcium pantothenate/10 ml. as previously described (1).

^b Per cent of incident light transmitted using the Evelyn colorimeter and the 660 filter, distilled water = 100. This reading obtained in 18 hr.

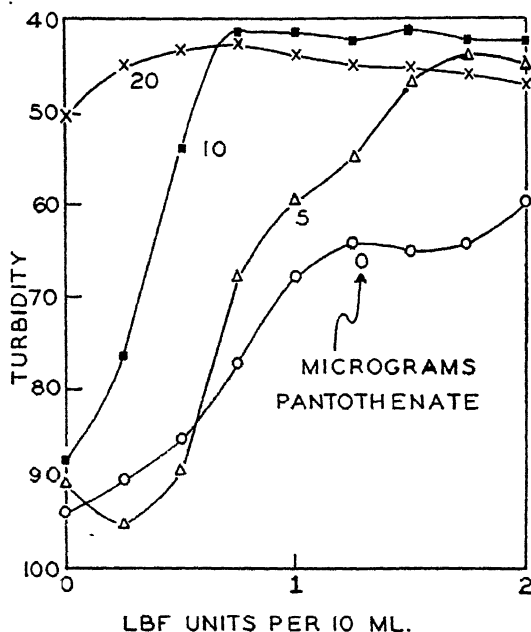


FIG. 1. Effect of various amounts of pantothenate on response of *Lactobacillus bulgaricus* to an LBF concentrate (potency 1000 units/mg.).

(20 µg.), there appears to be a small additional response to LBF. The LBF concentrate used here contained 0.0003 µg. of free pantothenate/LBF unit, an amount low enough to have no discernible effect on *L. bulgaricus*.

This concentrate, containing 0.0003 µg. of pantothenate/unit of LBF and 2000 units of LBF/ml., was found to promote growth of *Lactobacillus arabinosus* 17-5 and *Lactobacillus casei*. For *L. casei*, one unit of LBF was equivalent to 15.5 mµg. of pantothenate, while for *L. arabinosus* one unit gave growth corresponding to 1.0 mµg. of pantothenate.

The question arises as to which of these two substances, LBF or pantothenate, has the greater biological activity. For the three organisms, *L. bulgaricus* (Sarles' strain), *L. helveticus* 80, and *L. bulgaricus* 10, LBF concentrates are more potent than pantothenate, i.e., less solids are required to achieve growth. Concentrates of LBF have been prepared which contain 2000-10,000 units/mg. of solids and which give maximum growth of the above three organisms in amounts of .05 µg. and .01 µg./ml., respectively, whereas 1.0-1.3 µg. of pantothenate is required to give similar growth.

LBF concentrates containing no free pantothenate according to the yeast-assay method yield large amounts of β-alanine by hydrolysis with alkali. Assuming all the β-alanine arises from pantothenate contained in the LBF molecule, hydrolysis of various concentrates indicates one LBF unit contains 0.027 µg. of pantothenic acid; i.e., material of potency 1000 units/mg. contains 2.7% pantothenic acid.

EXPERIMENTAL METHODS

LBF was determined as described (1) except that Evelyn tubes were used instead of 18×150 -mm. tubes for growth of *L. bulgaricus*. This markedly improves the assay. *L. casei* (A.T.C.C. No. 7469) and *L. arabinosus* (A.T.C.C. No. 8014) were grown on the *L. bulgaricus* medium (1) from which the Tween 40 and oleic acid were omitted. This is essentially the medium and method of Roberts and Snell (4) which is suggested for pantothenate assay using *L. casei*. Concentrates mentioned were prepared from microbial growth medium employing charcoal adsorptions and elutions. Free pantothenate and β -alanine were determined by the method of Atkin *et al.* (5) using *Saccharomyces carlsbergensis* (A.T.C.C. No. 9080).

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Relation of the *Lactobacillus bulgaricus* Factor to
Pantothenic Acid and Coenzyme A^{1,2}

Partial purification and certain properties of an unidentified growth factor for *Lactobacillus bulgaricus* were reported previously (1,2). This factor, termed LBF, is required for rapid growth of many fastidious lactic acid bacteria (3). Concentrates of one form (2) of this factor have been obtained which are 25,000 times more active on a weight basis than a standard yeast extract, and which, though not pure, appear to consist principally of a single substance.

Evidence summarized below indicates that LBF is a bound form of pantothenic acid.

a) Large amounts of pantothenic acid replace LBF.² In a pantothenic-acid-free medium, 30 μ g. of calcium pantothenate or 0.4 μ g. of the LBF concentrate (potency 25,000) was required/10 ml. to permit maximum growth of *L. helveticus* 80. An addition of 8 μ g. of calcium pantothenate (insufficient by itself to permit growth) reduced the amount of LBF required from 0.4 μ g. (10 units) to 0.04 μ g. (1 unit). LBF is thus from 75 to 500 times more active than calcium pantothenate for this organism, depending upon the testing conditions.

¹ Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the U. S. Public Health Service and from Parke, Davis and Co., Detroit.

² W. L. Williams and co-workers have independently found that high levels of pantothenic acid replace LBF for these organisms (personal communication).

b) For *L. arabinosus*, LBF concentrates (potency 25,000) are about 25% as active as pantothenic acid in supporting growth; they are essentially inactive in replacing pantothenic acid for *Saccharomyces carlsbergensis*. The presence of "bound" pantothenic acid unavailable for *S. carlsbergensis* but available to *L. arabinosus* is thus suggested. In support of this conclusion, acid hydrolysis liberated approximately 30% of β -alanine (identified by paper chromatography and microbiological assay) from this concentrate, and digestion with a chicken liver enzyme liberated pantothenic acid equivalent to 65–75% by weight of the LBF concentrate used (Table I).

TABLE I
Relation of LBF to Pantothenic Acid and to Coenzyme A

| Sample | Treatment ^a | LBF content ^b | Activity in terms of calcium pantothenate ^b | |
|---|--|--------------------------|--|-------------------------|
| | | | $\mu\text{g./sample}$ | $\mu\text{g./unit LBF}$ |
| LBF conc. (250 units, 10 $\mu\text{g.}$) | None | 240 | 2.7 | 0.011 |
| LBF conc. (250 units, 10 $\mu\text{g.}$) | Intestinal phosphatase | 210 | 2.7 | 0.011 |
| LBF conc. (250 units, 10 $\mu\text{g.}$) | Chicken liver | 2.5 | 7.7 | 0.030 |
| LBF conc. (250 units, 10 $\mu\text{g.}$) | Intestinal phosphatase + chicken liver | 4.9 | 8.8 | 0.035 |
| Coenzyme A conc. (3 units) | None | 0.0 | 0.0 | — |
| Coenzyme A conc. (3 units) | Intestinal phosphatase | 60 | 1.1 | 0.018 |
| Coenzyme A conc. (3 units) | Chicken liver | 0.0 | 0.0 | — |
| Coenzyme A conc. (3 units) | Intestinal phosphatase + chicken liver | 0.0 | 2.2 | — |
| PAC ^c (300 $\mu\text{g.}$) | None | 0.0 | — | — |

^a The digestions of coenzyme A and of LBF concentrates with intestinal phosphatase and/or the acetone-dried chicken-liver preparation were carried out by published methods (5,6). Enzyme preparations, coenzyme A and PAC were supplied by Drs. V. H. Cheldelin, L. J. Teply, and D. E. Green. Preparations of coenzyme A and PAC contained approximately 1% and 0.4%, respectively, of bound pantothenic acid.

^b LBF was determined with *L. helveticus* 80 (1), and pantothenic acid with *L. arabinosus* (7). Assay values are corrected for blank values given by the enzymes alone.

^c Pantothenic acid conjugate.

c) Known bound forms of pantothenic acid were tested for LBF activity (Table I). Pantothenic acid conjugate (PAC) (4), coenzyme A (5), and the product formed from coenzyme A by digestion with chicken liver (5) were all inactive.³ Digestion of coenzyme A with intestinal phosphatase, however, liberated a substance with high LBF

³ At high levels, coenzyme A showed slight activity, but no higher than would be expected from its pantothenic acid content. Pantoic acid and β -alanine were inactive.

activity, and this substance, like LBF itself, was destroyed by digestion with chicken liver enzyme with the release of pantothenic acid. Intestinal phosphatase had no effect on the activity of the LBF concentrate.

Thus LBF contains bound pantothenic acid, and is probably either identical with or closely related to the product formed when coenzyme A is treated with intestinal phosphatase (5).

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Isolation of β -Glucuronidase of Calf Spleen¹

The proper understanding of the behavior of β -glucuronidase in nature requires a knowledge of the properties of the pure enzyme.

Several attempts to purify β -glucuronidase have been made (1-4). We describe in the following a method of purification of β -glucuronidase, using a commercially prepared defatted dry powder of calf spleen (Viobin Corporation).

Table I illustrates the essential features of our process. The two alkaline ammonium sulfate fractionations not only remove protein impurities but also acid material, probably of low molecular weight, which always would accompany the enzyme in an acid fractionation. The anion exchange has been carried out in order to avoid a dialysis (6) before the methanol fractionation. The low yields reflect the selection of only the purest fraction for the further purification and do not indicate major losses of β -glucuronidase.

The product obtained by this method behaves like a single protein on electrophoresis and may be considered to be pure calf spleen β -glucuronidase. The pure enzyme is 1200 times more active/mg. N than the starting material, and 1 mg. of this enzyme dissolved in 1 ml. of water hydrolyzes 0.165 mg. phenolphthalein glucuronide in 1 min. at 37°C. The maximum activity is exhibited at pH 4.5. The enzyme is a colorless product which is stable in solution. An absorption maximum is found at 277.5 m μ . The turnover number is calculated to be not greater than 40, assuming a molecular weight of 100,000.

¹ Aided in part by an institutional grant from the American Cancer Society, Inc., New York, N. Y., and the U. S. Public Health Service, National Institutes of Health, Cancer Institute, Washington, D. C.

TABLE I
Purification of β -Glucuronidase

| | pH | Fractionation limits | Purity ^a | Yield |
|---|-----|----------------------|---------------------|-------|
| | | % | | % |
| Dried defatted calf spleen | | | 5.9 | 100 |
| Crude extract | 5.0 | | 23.1 | 95 |
| 1st (NH ₄) ₂ SO ₄ precipitation | 4.5 | 0-60 satd. | 120 | 53 |
| 2nd (NH ₄) ₂ SO ₄ precipitation | 8.7 | 23.5-35 satd. | 520 | 24 |
| 3rd (NH ₄) ₂ SO ₄ precipitation | 8.7 | 33.5-37.7 satd. | 640 | 5 |
| Exchange of anions against citrate on Amberlite IR4B) | 4.7 | | 700 | 5 |
| 1st CH ₃ OH precipitation | 4.7 | 33-50 | 2300 | 2 |
| 2nd CH ₃ OH precipitation | 4.7 | 50-66 | 7000 | 1.5 |

^a Purity expressed in terms of μ g. phenolphthalein liberated (5)/hr./mg. enzyme.

The activity of a 0.01% solution of the enzyme is increased 100% by 0.3% desoxy-ribonucleate (7) and to a somewhat lesser degree by yeast nucleate.

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Book Reviews

The Alkaloids: Chemistry and Physiology. Vol. I. Edited by R. H. F. MANSKE, Dominion Rubber Research Laboratory, Guelph, Ontario, and H. L. HOLMES, University of British Columbia, Vancouver, Canada. Academic Press Inc., New York, N. Y., 1950. viii + 525 p. Price \$10.00.

At present the exploration of plant alkaloids has come to certain final conclusions. The important "classical" alkaloids conceal almost no secrets, after the most refractory problem, the constitution of the strychnos alkaloids, had found its final solution after more than a century of investigations.

It is, therefore, well-timed, as the editors of the present publication have undertaken, to compile this extensive material within the framework of a textbook. The few, mostly outdated monographs on alkaloids have become unsuitable in view of the importance of the subject.

The work will comprise four volumes, of which the first has been published. Preceding the systematic part is a short introduction about the occurrence, isolation, and purification of the alkaloids, by R. H. F. Manske, which is followed by a rather more detailed chapter by W. O. James dealing with the function of the alkaloids in plant metabolism and their biosynthesis.

The arrangement of groups in the special section does not quite correspond to the customary fashion. The insertion of the senecio alkaloids with their bicyclic pyrrolidine system between pyrrolidine and pyridine alkaloids can be justified although their relationship with the tropane group might better put them into that connection. But I should have liked to find the strychnos alkaloids later with the alkaloids derived from indol. It is regrettable, too, that the conclusion of the investigations into strychnine could not be included. Although periodic supplements are promised for the work, it should be considered whether in this case such an important omission should not be filled by a short addendum to the volume to be published soon.

The reviewer thinks that the material of the first volume has been collected with great thoroughness, the usual constants of the individual compounds being easily found in many clear tables. The reviewer can testify that the bibliography of the better known alkaloids is to be considered complete.

It would have been desirable if some references to the pharmacological action of the plant bases and the history of their research had been added. Probably it was not feasible to extend the limited scope of the book beyond the discussion of their chemistry.

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Biochemistry in Relation to Medicine. By. C. W. CARTER, Fellow of Queen's College, Oxford, and R. H. S. THOMPSON, Professor of Chemical Pathology, Guy's Hospital Medical School, University of London. Longmans, Green and Co., London, New York, Toronto, 1949. xi + 442 pp. Price \$5.00.

This book, designed for medical students, especially during their clinical years, combines the features of a short textbook and a manual for biochemical laboratory classes. It seems especially adapted for the medical students at the University of Oxford, England. For the teaching in this country, it would seem to be too short, at least to be used by the student as his only biochemical textbook. The choice as to what such an abridged text shall contain and what it shall omit is, of course, somewhat arbitrary, but fat and protein metabolism should be accorded more space. The most important sides of biochemistry and its clinical applications are, however, covered in the book including a short outline of the history and twenty pages of bibliography. The latter is a welcome deviation from the usual procedure of such students' textbooks. It may allow the student to deepen and enlarge his knowledge from a study of the original literature.

It may be questioned whether the inclusion of experimental techniques and methods in the body of the book is a good innovation, because it breaks up the continuity of thought in reading. The experimental part would be better placed at the end as an appendix. Only for the understanding of the pH, does this device seem appropriate because the discussion of the experimental details really adds to the understanding of the subject.

It is regrettable that in the chapter of plasma proteins the important separation of these proteins at the Harvard Medical Laboratories is not reviewed. This would have given a fine opportunity to show how a scientific investigation can contribute to the advancement of medicine.

The reviewer is somewhat disappointed by the rather numerous inaccuracies and obsolete statements included in the theoretical parts. A book published in 1949 should be up-to-date. Here are some such inaccuracies: On page 120 the formula for TPN is incorrect because the third phosphate is attached to ribose. It would be much better to give the formula of DPN which is more important. On page 129, oxalsuccinic acid, discovered in 1945 as intermediary between ketoglutaric and isocitric acids, is left out. On page 207, the scheme of dismutation is erroneous because the *L*- α -glycerophosphate is oxidized to dihydroxyacetone phosphate and not to *D*-glyceric aldehyde phosphate. Likewise, an erroneous statement is made on page 213 where the oxidation-reduction is ascribed to "an enzyme of the mutase type in conjunction with cozymase." This phrase is misleading because in the dismutation two enzymes, an oxidase and reductase, are concerned and the mutases (like phosphoglucosemutase) are enzymes which shift the phosphate group. These examples taken at random could be easily increased. It is to be hoped that in future editions the accuracy of the statements will be thoroughly reviewed.

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Annual Review of Biochemistry, Vol. XVIII. Editor: J. MURRAY LUCK. Annual Reviews, Inc., Stanford, Calif., 1949. vi + 738 pp. Price \$6.00.

The arduous task of preparing reviews of the papers published during 1948 in twenty-three fields of biochemistry has found expression in the appearance of Vol. 18 of the "Annual Review of Biochemistry." "The writing of reviews of one sort or

another is becoming a literary and intellectual experience which falls to the lot of almost every scientist sooner or later" say the editors. But the writing of such reviews is also a grave responsibility, not to be undertaken lightly, for the research worker today, can scarcely hope to cope adequately with the literature in his field without reference to, and guidance from, these reviews. Moreover, the young research worker is apt to be swayed by the opinion of the reviewer and to be impressed by the manner in which facts are marshaled to present a thesis or a fresh point of view.

The *Annual Review* is now a stand-by of all research workers in the biochemical field and all readers must feel a great debt of gratitude to the reviewers who so painstakingly and conscientiously present the data accumulated in the past year in as clear and unprejudiced a manner as possible. The *Annual Review* is now a prolific parent for it has given rise to offspring which promise to be as sturdy, and possibly as fruitful, as the parent; namely: The Annual Reviews of Physiology, and of Microbiology, and of Plant Physiology, the last being the latest of the progeny.

The problem of literature citation is mentioned by the editors in their preface and they state that they would welcome the advice of their readers in this important, and indeed essential, matter. The writer feels that the present method of giving numbers to text references which are collected in order at the end of the article is logical and adequate, and that there is no real necessity to place references in alphabetical order at the end of the article, so long as the volume has an author index. The writer feels, however, that since so many articles are of joint authorship, *every* author should be referred to by name at least once in the text, that his name should not be obscured by *et al.* in the text and find only belated recognition in the bibliography. Either authors' names should not be mentioned at all in the text, which would be most impracticable and inadvisable, or they should all be mentioned at least once. The extra amount of space thus taken up would surely not be so large as to outweigh the advantage of the junior members of a research team finding their names recognized in the text.

The articles contained in Volume 18 of the *Annual Review* are of a high standard, and, so far as the fields with which the writer is familiar are concerned, are most competently handled. The enzyme field is divided as usual into proteolytic, and non-oxidative non-proteolytic sections and these are dealt with by Drs. E. L. Smith and K. Myrback, respectively. The vitamin field is divided into the classical fat-soluble, and water-soluble sections, these being handled by Dr. P. L. Harris, and by Drs. E. L. R. Stokstad and T. H. Jukes, respectively. The steadily advancing subject of metabolic inhibitors is reviewed by Dr. R. J. Winzler, and antibiotic biochemistry is in the hands of Drs. O. Wintersteiner and J. D. Dutcher. A most interesting review is that by Dr. V. B. Wigglesworth on insect biochemistry, no review of this subject having been made since 1940. Hormone chemistry, the chemistry of fats, proteins, carbohydrates, and phosphorous compounds; the biochemistry of minerals, plants, and of neoplasms; all these are dealt with by acknowledged experts in their subjects.

J. H. QUASTEL, Montreal, Canada

Advances in Protein Chemistry, Vol. V. Edited by M. L. ANSON, J. T. EDSALL and K. BAILEY. Academic Press Inc., New York, N. Y., 1949. ix + 481 pp. Price \$7.50.

All those who are more-or-less interested in proteins await with impatience the annual issue of "Advances in Protein Chemistry." They hope to find in it precise

information in the field of their own research, and also an improvement of their general knowledge. By the ability of its contributors and the diversity of its subjects, the fifth volume of this series seems to meet perfectly this twofold expectation.

It includes the following eight reviews:

The Synthesis of Peptides by JOSEPH S. FRUTON. 75 pp., 19 tables, 305 refs. The identification of the partial-hydrolysis products of proteins and the systematic study of endo- and exopeptidase specificity nowadays implies obtaining an ever-increasing number of pure peptides with a definite structure. This review consists of two parts. In the first one, we find a general description of every method of synthesis heretofore suggested, with a comprehensive discussion of their respective advantages and the results obtained so far. The most important of them [those using the α -halogen (or α -azido) acyl halides, the azlactones, and the carbobenzoxy, phthalyl, or toluenesulfonyl derivatives] receive special attention. In the second part, the author shows which he considers the best methods for the preparation of special peptides. The chief properties (melting point, specific rotation, solubility) of the peptides and of some of the intermediary products are given in many tables.

Amino-Acid Composition of Purified Proteins by G. R. TRISTRAM. 72 pp., 37 tables, 214 ref. The School of Biochemistry in Cambridge has become a very active center for the study of protein structure. G. R. Tristram's review, coming after those of Chibnall (1946) and Brand (1946), is therefore a very welcome one.

The main interest of this review clearly lies in its numerous tables. A first series is devoted to a comparative survey of the results given by different analytical methods for the determination of 19 amino acids in some "standard" proteins. A fair agreement exists between such results, which shows that important progress has been made within the last few years. Another series gives the amino-acid composition of 24 proteins with, for each amino acid, the percentage (w/w), the number of residues in 10^6 g. (or in one mol) of protein, and also some general information easily derived from the analysis, such as mean residue weight, proportions of polar and nonpolar groups, minimum molecular weight of the protein, etc.

When writing this review, the author was obliged to classify a considerable amount of bibliographic material. It is therefore not surprising that he has mentioned only English-language literature. We may perhaps also point out that the analytical methods have not yet reached a sufficient accuracy to allow the use of two decimals for the amino-acid percentages.

Biological Evaluation of Proteins by JAMES B. ALLISON. 45 pp., 6 tables, 10 figs., 134 refs. It seems to be now well recognized that the chemical composition of a protein cannot be used as the only criterion of its biological value. This latter depends on the ease with which the essential amino acids or peptides are liberated from it by the digestive enzymes. It also varies with the animal and the more-or-less saturation of its protein stores. To get an accurate idea of this biological value it is therefore necessary to measure the protein minima for nitrogen equilibrium, the nitrogen balance index of nitrogen intakes (or absorbed nitrogen), and to observe the protein efficiency concerning growth and tissue regeneration. J. B. Allison's review has the advantage of discussing these rather complicated notions in a clear and systematic way.

Milk Proteins by THOMAS L. McMEEKIN and B. DAVID POLIS. 26 pp., 6 tables, 148 refs. In this short review, the authors seem to have made the most of a very intricate subject. They describe the preparation and composition of α - and β -cow's casein, as well as Mellander's work on human casein. They give a good discussion of the properties of β -lactoglobulin. But the last chapter dealing with the enzymes of milk is in too abstract a form to be of sufficient interest.

Plant Proteins by J. W. H. LUGG. 74 pp., 7 tables, 395 refs. Though they are of the greatest interest in animal nutrition, plant proteins have so far not been the object of much study. Their special properties, their complexity and the difficulties we meet when trying to fractionate them systematically have discouraged research work in this field. We are therefore grateful to J. W. H. Lugg for his comprehensive review which will certainly bring plant proteins back in favor with many of us.

Synthetic Fibers Made from Proteins by HAROLD P. LUNDGREN. 46 pp., 21 figs. 174 refs. The first part of this paper shows how a protein fiber can be made. All these processes are directly connected with the fundamental problems of protein structure. It is highly interesting to observe these problems from a practical and industrial point of view.

Protein Chemical Aspects of Tanning by K. H. GUSTAVSON. 68 pp., 4 tables, 4 figs., 222 refs. We find here another technical side of protein chemistry. After a sound discussion of the general properties of collagen, the author gives a detailed study of the usual tanning processes from a point of view which is as scientific as possible. Unfortunately, our knowledge concerning the action of many tanning agents on the free groups of proteins is still very far from being complete.

Proteins, Lipides and Nucleic Acids in Cell Structure and Functions by ALBERT CLAUDE. 27 pp., 64 refs. Under this quite general title the author tries to consider proteins (as well as other substances), no longer as chemicals "*in vitro*," but as "*in vivo*" cellular components with a definite spacial organization.

P. DESNUELLE, Marseille, France

Erratum

In the Letter to the Editors by Waelsch *et al.* entitled, "The Enzyme-Catalyzed Exchange of Ammonia With the Amide Group of Glutamine and Asparagine," which appeared in Volume 27, Number 1, p. 237, the table should read as follows:

TABLE 1

Enzymatic Exchange of Ammonia with the Amide Groups of Glutamine and Asparagine

| Addition | Enzyme extract | | |
|--|----------------|---|----------|
| | Absent | Present | |
| | | N ¹⁵ found in amide group | Exchange |
| | | Excess atom—% | per cent |
| <i>μmoles</i> | | | |
| Glutamine ^a 120 NH ₄ Cl ^d 260 | 0.118 (1) | 1.88 (3) | 5.0 |
| Glutamine ^b 240 NH ₄ Cl ^d 680 | | 2.74 (4) | 7.7 |
| Asparagine ^c 300 NH ₄ Cl ^d 500 | 0.018 (2) | 1.39 (5) | 4.3 |

^a Three ml. enzyme solution or water, total volume 12 ml.

^b Nine ml. enzyme solution, total volume 20 ml.

^c Four-tenths ml. of enzyme solution or water, total volume 4 ml. Enzyme solution contained 1 mg. undialyzable N/ml.

^d Thirty-two atom-% excess N¹⁵.

N¹⁵ atom-% excess in NH₃ of last distillate before liberation of amide group: (1) 0.235, (2) 0.040, (3) 0.296, (4) 0.275, (5) 0.018.

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The Application of Paper-Partition Chromatography to the Analysis of Chloramphenicol (Chloromycetin)¹ and Decomposition Products²

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INTRODUCTION

The paper-partition chromatography procedures developed by Consden, Gordon, and Martin (2) have been found useful in the separation of many classes of compounds (3,5-10,14,22-24). It was found desirable, during a series of recent investigations on the interrelationships of enzymatic systems and chloramphenicol, to adapt the paper-chromatography technique for the separation and identification of chloramphenicol and related decomposition products formed by enzymatic or chemical destruction of the drug.

In order to adapt the method to the study of compounds having a structure somewhat similar to chloramphenicol, a color reaction had to be developed to locate the positions of the compounds on the paper strips. A study of the wide range of compounds which could be formed revealed that no single test could be used for all compounds. A consideration of the functional groups which might be encountered indicated that one might expect to find decomposition products containing nitro, aromatic amino, aliphatic amino, ketone, aldehyde, hydroxyl, and carboxyl groups. By employing specific chemical tests for each of these functional groups, the position of each decomposition product on the paper strip could be ascertained. The present paper gives some of the observations which have been obtained using these tests.

¹ Parke, Davis and Company trade name for chloramphenicol.

² Reported in part at the 34th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1950.

CHROMATOGRAPHY

Whatman No. 1 filter paper strips 2×55 cm. or sheets 45×55 cm., respectively, were used in the orthodox "descending" method (2) or with the more recent "ascending" method (22). Approximately 0.05 ml. of the solution containing approximately 20 μ g. of the compound was applied 8 cm. from one end of the filter paper in such a manner that the area covered by the solvent did not exceed 0.5 cm. in diameter. When the original area was larger than this, the sharpness of the developed chromatogram was somewhat impaired.

After air-drying, the paper was developed at 30°C. in a gas-tight chamber with a water-saturated *n*-butanol mixture containing 2.5% acetic acid. The atmosphere within the chamber was kept saturated with respect to both water and *n*-butanol. After the solvent had run the desired distance (35–40 cm.), the paper was removed and the solvent front marked. The paper was dried at room temperature and the position of the individual compounds determined by means of the methods described below. In routine practice it has been found convenient to run a series of paper strips of each compound and develop several strips by each of the methods described.

METHODS FOR DETECTING CHLORAMPHENICOL
AND RELATED COMPOUNDS*Compounds Containing Aryl Amino Groups*

The position on the paper strips of those decomposition products of chloramphenicol which contain an aryl amino group in place of the nitro group present in chloramphenicol were detected by spraying the air-dried paper with a solution of *p*-dimethylaminobenzaldehyde containing 1 g. of *p*-dimethylaminobenzaldehyde dissolved in a mixture of 30 ml. of ethanol, 30 ml. of concentrated hydrochloric acid, and 180 ml. of *n*-butanol. After air-drying the paper, yellow spots appear in those locations containing the aryl amino decomposition products. This test is based on the condensation of an aromatic aldehyde with a primary amine. Both aromatic and aliphatic amines can take part in this condensation, but only the aromatic amines produce colored complexes.

Compounds Containing a Nitro Group

Chloramphenicol and related compounds which contain a nitro group can be identified by reducing the nitro group to an aryl amino group. The aryl amino group can then be identified by the procedure described above. The reduction of the nitro group was brought about by spraying the paper with a solution of stannous chloride prepared by mixing 3 ml. of standard solution of stannous chloride (15%) with 15 ml. of concentrated hydrochloric acid and 180 ml. of water. The solution should be prepared fresh each time it is to be used. After spraying, the strips were air-dried and sprayed with the *p*-dimethylaminobenzaldehyde solution. On air-drying the strips, the areas containing the nitro compounds appeared as yellow spots. The background is at the beginning a light yellow and the spots are clearly visible, but after standing for some time the background gradually deepens in color. It is therefore necessary to mark the spots as soon as they are developed.

Compounds Containing an Aliphatic Amino Group

The main hydrolytic product of chloramphenicol is the amino base which possesses a primary aliphatic amino group with an adjacent primary hydroxyl group. This combination of functional groups will react with the ninhydrin reagent in the same manner as do amino acids to give a characteristic colored complex. The ninhydrin reagent can, therefore, be used to detect such compounds on the paper strips. The ninhydrin reagent was prepared by diluting the standard ninhydrin reagent described by Moore *et al.* (13) with an equal volume of *n*-butanol. The paper strips were sprayed with the reagent and heated for 3 min. at 100°C. The areas containing the reactive compounds appear as purple or pink spots. The heating period must be restricted to 3 min., as under these conditions only the most reactive compounds such as amino acids and chloramphenicol breakdown products with an amino group adjacent to a hydroxyl, formyl or carboxyl group will give the characteristic colored complex. If the samples are heated for longer periods (>20 min.) many other aliphatic amines will react.

Compounds Containing a Formyl or Carbonyl Group

Those compounds containing a formyl or carbonyl group were identified by spraying the paper with a solution of ammoniacal silver nitrate. After maximum color development the paper was rinsed with a 5% solution of sodium thiosulfate and then with water. The spots appear as dark brown areas. The ammoniacal silver nitrate solution was prepared by adding 10 ml. of 0.1 *N* silver nitrate solution to 5 ml. of 10% sodium hydroxide solution and then adding concentrated ammonium hydroxide drop by drop until the precipitate of silver oxide had just dissolved.

During the course of these investigations it was found that certain degradation products of chloramphenicol containing a formyl (aldehyde) or carbonyl (ketone) group would give specific color reactions with 1.0 *N* sodium hydroxide or unheated benzidine reagent (8). By using these tests in combination with the ammoniacal silver nitrate test it was possible to distinguish the individual compounds in a mixture having the same R_F values.

RESULTS AND DISCUSSION

In Table I are listed some of the various degradation products of chloramphenicol which have been studied by the methods described. The R_F values listed are the average of 10 or more runs using the "descending" method of development. The plus marks indicate which tests are applicable for locating the compounds on the paper strip. The R_F values given in the table were obtained from experiments carried out with authentic samples added to broth cultures. The R_F values obtained for the pure compound in aqueous solution were generally about 5% higher.

Variations in the R_F value in repeated identical runs were found to be less than $\pm 5\%$. The factors responsible for these variations have been discussed in detail by Consden *et al.* (2). Variations in the con-

TABLE I

| Name | Structure | R_F | Nitro test | Aryl amine test | Ninhydrin test | Benzidine test | $AgNO_3NH_3$ test | NaOH test |
|---|-----------|-------|------------|-----------------|----------------|----------------|-------------------|-----------|
| <i>p</i> -Aminophenylserine | | 0.08 | + | + | + | | | |
| 1-(<i>p</i> -Aminophenyl)-2-amino-1,3-propanediol | | 0.12 | + | + | + | | | |
| Ethanolamine | | 0.25 | | | | | | |
| α -Amino- β -hydroxy- <i>p</i> -nitropropionophenone-HCl | | 0.36 | + | | | | + | + |
| 1-(<i>p</i> -Nitrophenyl)-2-amino-1,3-propanediol | | 0.15 | + | | + | | | |
| 1-(<i>p</i> -Aminophenyl)-2-dichloroacetamido-1,3-propanediol | | 0.69 | + | + | | | | |
| <i>p</i> -Aminobenzoic acid | | 0.78 | + | + | | | | |
| <i>p</i> -Nitrobenzoic acid | | 0.82 | + | | | | | |

TABLE I—Continued

| Name | Structure | R _T | Nitro test | Aryl amine test | Ninhydrin test | Benzidine test | Ag\O ₂ \H ₂ test | NaOH test |
|---|-----------|----------------|------------|-----------------|----------------|----------------|--|-----------|
| <i>p</i> -Aminobenzaldehyde | | 0 84 | + | + | + | + | + | + |
| Formaldehyde | | 0 85 | + | + | + | + | + | + |
| α -Acetamido- β -hydroxy- <i>p</i> -nitropropiophenone | | 0 86 | + | + | + | + | + | + |
| α -Acetamido- <i>p</i> -nitroacetophenone | | 0 87 | + | + | + | + | + | + |
| Chloroamphenicol | | 0 89 | + | + | + | + | + | + |
| α -Dichloroacetamido- β -hydroxy- <i>p</i> -nitropropiophenone | | 0 95 | + | + | + | + | + | + |
| <i>p</i> -Nitrobenzaldehyde | | 0 95 | + | + | + | + | + | + |

centrations of the compound present appeared to produce no significant change in the observed R_F values. Because of the many factors influencing the R_F value, great significance should not be placed upon its absolute magnitude. The more important consideration lies in the relative movement of the various compounds and their characteristic reactions with the test reagents. When chromatographed side by side in equal quantities, this relative movement is constant.

The farther a compound moved in the chromatogram, the larger was the spot formed. In the case of compounds moving a short distance, *e. g.*, R_F 0.10, an elliptical spot of approximately 0.9×0.5 cm. was produced, while with a similar quantity of the compounds which moved approximately to R_F 0.50, a spot of 1.5×0.5 cm was produced. The tendency to form larger spots with farther migration seemed to be also characteristic of certain of the compounds. In all cases the center of the spot was used in calculating the R_F values.

The procedures described for determining the presence of nitro and aryl amino groups can be used either with the pure chemical compound or with compounds present in bacterial cultures after the bacterial cells have been removed by Seitz filtration. It is not necessary to completely remove all traces of proteins and amino acids that might be present.

The quantity of reducing agent used in this test is sufficient to reduce the nitro groups present even if a considerable quantity of other reducible substances are also present. The coupling reaction will proceed in the presence of the reducing agent so it is not necessary to destroy the stannous chloride before coupling.

The ninhydrin reagent can be used to detect those degradation products of chloramphenicol which have a free aliphatic amino group with an adjacent primary hydroxyl group. This combination of functional groups is formed by the hydrolysis of the amide linkage present in chloramphenicol. The ninhydrin reaction is, however, not specific for this one combination of functional groups. Any compound which contains a primary aliphatic amino group with an adjacent primary hydroxyl, formyl, or carboxyl group may react with the ninhydrin reagent during a short heating period (3 min.). Therefore, in studying the degradation products of chloramphenicol which might be present in biological solutions containing proteins and amino acids, it is necessary to remove by extraction the chloramphenicol decomposition products and determine them separately. This can generally be done with ethyl acetate, *n*-butanol, or a mixture of *n*-butanol-benzene (1:1). The solubility

properties of the various compounds can be ascertained by referring to the chemical studies on these compounds (1,4,11,12,15).

In studying those compounds which react with the ninhydrin reagent, a distinct color reaction was observed with several of the compounds. For example, 1-(*p*-nitrophenyl)-2-amino-1,3-propanediol gave a purple color with the reagent while 1-(*p*-aminophenyl)-2-amino-1,3-propanediol produced a salmon-colored spot. Other compounds gave spots which appeared almost black or dark brown in color. Also, a great variation in the rate of reaction was noticed. Compounds containing the characteristic amino acid groups reacted faster than the compounds containing the aliphatic amino group with an adjacent hydroxyl group. Both of these compounds reacted with the ninhydrin reagent within 3 min. and could easily be distinguished from the other aliphatic amines which reacted only after prolonged heating periods.

The ammoniacal silver nitrate test can be used to detect degradation products of chloramphenicol which contain either a formyl or carbonyl group, providing these compounds have been separated from the other substances such as sugar, sodium chloride, *etc.*, which might be present in the biological solutions and which would react with this reagent.

Most of the degradation products of chloramphenicol which do react with the ammoniacal silver nitrate reagent unfortunately have approximately the same R_f values. Chemical tests must therefore be resorted to in order to determine exactly which compounds might be present at this position. Certain of the decomposition products of chloramphenicol containing a formyl (aldehyde) or carbonyl (ketone) group have characteristic reactions which can be used to identify them. For example, *p*-nitrobenzaldehyde, α -acetamido- β -hydroxy-*p*-nitropropiophenone and α -acetamido-*p*-nitroacetophenone gave positive reactions when the paper strips were developed with ammoniacal silver nitrate and all three compounds have approximately the same R_f values. They can be easily distinguished, however, by developing the paper strips with either 1.0 *N* sodium hydroxide or with the benzidine reagent.

With 1.0 *N* sodium hydroxide, α -acetamido- β -hydroxy-*p*-nitropropiophenone gave a bright yellow spot which rapidly faded, while α -acetamido-*p*-nitroacetophenone produced a bright rust colored spot which persisted for some time. However, with *p*-nitrobenzaldehyde no color change can be detected. When the paper strips are developed with benzidine reagent, *p*-nitrobenzaldehyde reacts instantaneously to give

a bright yellow spot, while the other two compounds produced no significant color.

The reactions described above make it possible to differentiate *p*-nitrobenzaldehyde and α -acetamido- β -hydroxy-*p*-nitropropionophenone and α -acetamido-*p*-nitroacetophenone even though the compounds have the same R_F values.

The procedures outlined have been found to be very useful tools in studying the formation and decomposition of chloramphenicol providing certain fundamental precautions are observed. It should be remembered that certain of the decomposition products of chloramphenicol possess a yellow color; therefore, in using the nitro or aryl amine tests with paper strips containing yellow-colored compounds, a positive test is indicated by a darkening of color. Furthermore, the aryl amine test should be performed before the nitro test, as the nitro test is based on the reduction of the compounds to aryl amine products which are then tested for. It is, therefore, necessary to know if aryl amine compounds are already present and their location on the paper strip.

In many cases a variety of compounds are encountered which have approximately the same R_F value. In these cases a single separation does not permit a clear differentiation of the components. It has been found advisable under this circumstance to chromatograph the samples first on large sheets, then section the sheets, extract the compounds and rechromatograph the material.

In the biochemical studies (21) in which 15–20 decomposition products of chloramphenicol were encountered in the broth solutions, a preliminary separation using a liquid–liquid extraction procedure (21) was employed to separate the material into basic, acidic and neutral components. Then by chromatographing the material several times an adequate separation and identification of the various decomposition products was possible.

ACKNOWLEDGMENTS

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SUMMARY

1. The method of partition chromatography on paper has been applied to separation of chloramphenicol and related degradation products.

2. New chemical methods are described for the identification of aromatic nitro and amine compounds.

3. Additional chemical tests are described which permit the identification of the individual compounds even though they may have the same R_F values.

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Tautomeric Conversion of Xanthopterin¹

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INTRODUCTION

Since xanthopterin was discovered as one of the pigments in butterfly wings (1,2), this compound has been shown to be present in the organs of several species of animals (3-8). Furthermore, it has been demonstrated that a xanthopterin-like substance, "proxanthopterin", is excreted in the urine of the horse (3), rabbit (3,9), rat (9), and man (3,10).

The physiological significance of xanthopterin remained rather obscure for a long time until it was discovered that the vitamins belonging to the folic acid group are derivatives of 2-amino-4-hydroxypteridine, as is xanthopterin (11). Recent investigations reveal that the administration of folic acid to man is followed by an increased excretion of proxanthopterin in the urine (10).

The constitution of xanthopterin has been shown to be 2-amino-4,6-dihydroxypteridine (12-14); but, so far, only scanty information has been available as to the tautomeric and the acid-base properties of this compound.

A detailed study of the influence of hydrogen-ion concentration on the optical properties of xanthopterin is presented in this report. The fluorescence and the ultraviolet absorption change in a characteristic way with the pH, and some of these phenomena may be explained by assuming a tautomeric equilibrium between an enol and a keto form of xanthopterin. The experiments suggest, furthermore, that only the enol form is oxidized to leucopterin in the presence of the enzyme xanthopterin oxidase.

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EXPERIMENTAL AND RESULTS

Apparatus and Chemicals

The fluorescence measurements were performed with a Farrand microphotofluorometer (15) containing as primary light filter a Corning filter 5860 (transmitting ultraviolet radiation at 365 m μ). The fluorescent light passed a combination of Corning filters 4308 and 3389 with maximal transmission at 300 m μ . Immediately before each reading, the sensitivity was adjusted to give a reading of 100 with a standard solution containing 0.014 μ g. of quinine sulfate/ml. in 0.1 *N* sulfuric acid.

For the measurement of ultraviolet absorption a Beckman model DU quartz spectrophotometer was used.

In the experiments, two different samples of synthetic xanthopterin were employed. One sample, which was supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company, had been synthesized by the classical Purrmann procedure (16). The other sample of xanthopterin was furnished by Dr. G. H. Hitchings of Burroughs Wellcome and Company, who had used his own synthetic method (17). The two samples of xanthopterin showed identical optical properties.

Stock solutions of xanthopterin in 0.1 *N* sodium hydroxide were stable for several months when kept in the refrigerator.

The concentrate of xanthopterin oxidase was prepared according to Ball (18) as modified by Kalckar, Kjeldgaard, and Klenow (19).

Fluorescence of Xanthopterin

The fluorescence of xanthopterin varies markedly with the hydrogen-ion concentration. Lowry *et al.* (20) pointed out that, in acid solution, a number of anions decrease ("quench") the fluorescence, and they also observed that, when a solution of xanthopterin is diluted with phosphate buffer, the fluorescence does not immediately reach a stable value but changes somewhat for a period of minutes.

This slow change in fluorescence, however, is only observed when the pH of the xanthopterin solution is adjusted to about pH 7. (See Table I.)

The change of hydrogen-ion concentration from pH 13 to pH 7 or pH 6 apparently gives rise to two different processes: First, reaction *a*, an instantaneous decrease ("quenching") of the fluorescence (at pH 7 from 149 to 96), followed by reaction *b*, a slower decrease (at pH 7 from 96 to 80). Similar changes were observed when the phosphate buffer was substituted by borate or maleate buffers.

The "quenching," reaction *a*, seems to be due to a stronger fluorescence-depressing power of the anions at lower pH values. Table II shows that at a fixed pH this anion quenching is, in fact, an instan-

TABLE I

An alkaline solution of xanthopterin was diluted at zero time to a concentration of 200 $\mu\text{g.}/\text{ml.}$ with buffer solution, and the fluorescence was read at intervals.

Buffer solutions: 0.2 *M* phosphate buffer, pH 11.0; 0.2 *M* phosphate buffer, pH 7.0; 0.2 *M* phosphate buffer, pH 6.2; 0.2 *M* acetate buffer, pH 3.4.

| Time, min. | | 0.5 | 2 | 6 | 15 | 40 | 60 | 100 | 150 |
|---------------------------|------|--------------|-----|-----|-----|-----|-----|-----|-----|
| | pH | Fluorescence | | | | | | | |
| pH changed from 13 to: | 11.0 | 149 | 150 | 148 | 149 | 150 | 150 | 149 | 149 |
| | 7.0 | 96 | 96 | 95 | 89 | 85 | 83 | 80 | 81 |
| | 6.2 | 40 | 37 | 35 | 30 | 25 | 24 | 24 | 24 |
| | 3.4 | 13 | 12 | 13 | 13 | 13 | 12 | 12 | 13 |

taneous reaction. The data of this experiment also confirm the observation of Lowry *et al.* (20) of a linear relationship between the reciprocal of the fluorescence and the concentration of the quenching anion.

The *slow* change in fluorescence at pH 7 and pH 6, thus, cannot be a quenching phenomenon, but must be due to a more sluggish conversion of xanthopterin or to a slow change of its properties. Since, at pH 7, reaction *b* is quite slow, while the change in fluorescence due to quenching is instantaneous, it is possible to observe the true progress of reaction *b* at different hydrogen-ion concentrations by using the procedure shown in Fig. 1. The quenching effects are eliminated here, and only changes due to reaction *b* are recorded.

When the hydrogen-ion concentration is changed from a definitely alkaline or a definitely acid level to pH values below 5 or above 9, respectively, the changes in fluorescence, due to reaction *b*, are maximal

TABLE II

Neutral solutions of xanthopterin were diluted at zero time to a concentration of 200 $\mu\text{g.}/\text{ml.}$ with 0.02 *M*, 0.1 *M*, and 0.2 *M* phosphate buffer solutions of pH 7.0, and the fluorescence was read at intervals.

| Time, min. | 0.5 | 5 | 30 | 60 |
|-------------------------|-----|-----|-----|-----|
| Fluorescence | | | | |
| 0.02 <i>M</i> Phosphate | 176 | 173 | 173 | 175 |
| 0.1 <i>M</i> Phosphate | 124 | 123 | 125 | 125 |
| 0.2 <i>M</i> Phosphate | 80 | 80 | 81 | 80 |

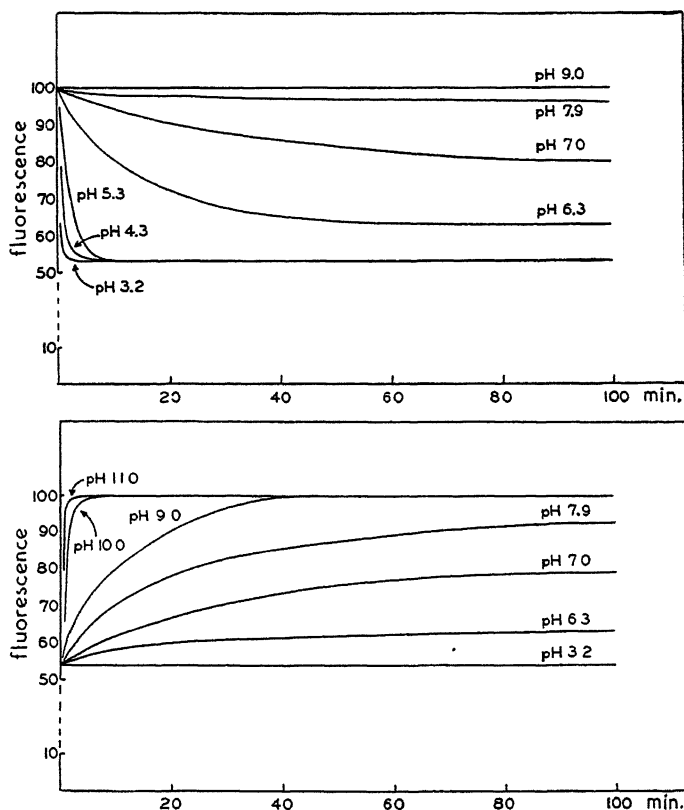


FIG. 1. Xanthopterin in 0.1 *N* sodium hydroxide (Fig. 1A) or in 0.02 *M* acetate buffer, pH 3 (Fig. 1B) was adjusted at zero time to various pH values between pH 11 and 3 by diluting with appropriate buffer solutions. At intervals samples were withdrawn, and the fluorescence was read immediately after dilution to a concentration of 200 $\mu\text{g./ml.}$ with 0.2 *M* phosphate buffer, pH 7.0. Abscissa: Time in minutes. Ordinate: Fluorescence.

and take place rapidly. At pH values between 5–9, the changes are slower, the final, stable fluorescence values are intermediate and the same, whether the initial solution of xanthopterin is acid or alkaline. It is seen from Fig. 2 that the change in fluorescence is much slower at pH 7.0 than at any higher or lower pH value.

In Fig. 3 the final, stable fluorescence values are plotted against the pH.

The phenomena observed may be explained by assuming that there is an equilibrium between a fluorescent form of xanthopterin and a less fluorescent or nonfluorescent conversion product, and that the ratio between these two depends upon the hydrogen-ion concentration. As this new substance might be a modified form of xanthopterin it will be convenient to designate the two compounds X and X^0 , X being the more fluorescent compound predominating at pH 9 and X^0 the less fluorescent conversion product. If X^0 is assumed to be nonfluorescent,

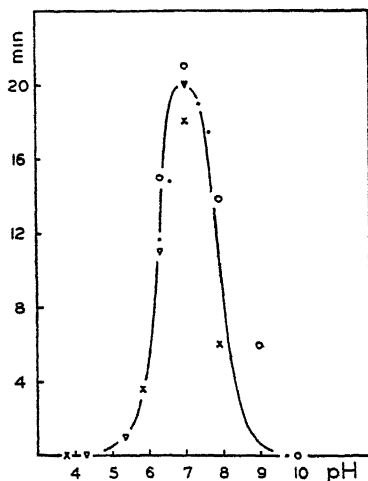


Fig. 2. Time required for attaining 50% of the final change in fluorescence (Fig. 1) or in optical density at 390 $m\mu$ (experiment described in Table IV). Abscissa: pH. Ordinate: Time in minutes. ∇ : Initial xanthopterin solution alkaline, Fig. 1A. \circ : Initial xanthopterin solution acid, Fig. 1B. \times : Initial xanthopterin solution alkaline, Table IV. \bullet : Initial xanthopterin solution acid, Table IV.

Fig. 3 will show that, at hydrogen-ion concentrations more alkaline than pH 9, all of the xanthopterin is in the form of X ; whereas at pH 7.0 the ratio X/X^0 is about 4:1, and at pH values 5 to 3, the two compounds occur in approximately equal amounts. (The behavior of xanthopterin at pH values below 3 will be discussed later.)

The experiments described were all performed at room temperature (20–22°C.). It can be shown that at lower temperatures the equilibrium between the two forms of xanthopterin is displaced in favor of compound X^0 , while heating brings about a transformation of X^0 to X .

An alkaline solution of xanthopterin was diluted to a concentration of 200 $\text{m}\mu\text{g./ml.}$ with 0.2 M phosphate buffer, pH 7.0. The fluorescence reading fell from 100 to a constant value of 80 in about 2 hr.

After heating of the solution for 1 min. in a boiling water bath and subsequent rapid cooling to room temperature, the reading was again 100, and it returned to 80 after 2 hr. at room temperature. Heating to 100°C. for 10 min. or boiling in a free flame for a short time caused no additional increase in fluorescence.

Cooling to 0°C. for 10 min. followed by rapid heating to room temperature did not change the fluorescence. The reading after 30 min. at -12°C. was 77, after 6 hr. at -12°C. 59, and after 6 hr. in liquid air 62, after rapid heating to room temperature.

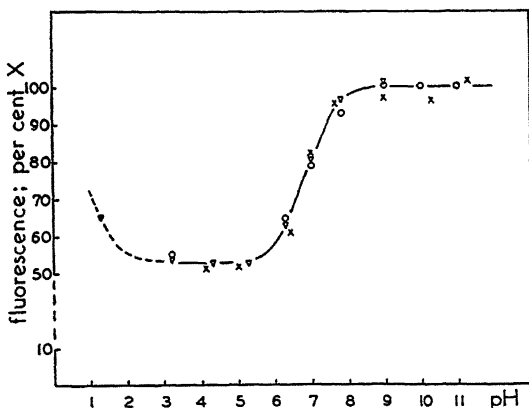


Fig. 3. Final, stable fluorescence values from Fig. 1, and per cent of compound X, plotted against pH. Abscissa: pH. Ordinate: Fluorescence; per cent X. ▽: From Fig. 1A. ○: From Fig. 1B. ×: Per cent X, calculated as described under "example of calculation," page 22.

Not only is the equilibrium influenced by temperature, but also the rate with which this equilibrium is attained. The rate becomes greater as the temperature increases. (Data not given here.) This explains the observation that the fluorescence, after freezing in liquid air, is higher than after cooling for the same length of time to only -12°C. Apparently at -180°C. the rate of the transformation decreases so rapidly that only a limited conversion of X to X^0 is observed.

It was essential to take all readings at the same temperature (20 – 22°C.), because the fluorescence of xanthopterin depends upon the temperature as shown in Fig. 4. These changes with temperature are instantaneous.

The conversion, $X \rightleftharpoons X^0$, was investigated both aerobically and anaerobically and was shown to be independent of the presence of oxygen. Ultraviolet irradiation was also without influence.

It can be shown that, while xanthopterin in the fluorescent form is oxidized to nonfluorescent leucopterin in the presence of xanthopterin oxidase (21,22), compound X^0 is not attacked.

An alkaline solution of xanthopterin was diluted to a concentration of 200 $\mu\text{g./ml.}$ with 0.2 M phosphate buffer, pH 7.0. The fluorescence reading fell from 100 to a constant value of 80 in about 2 hr.

At zero time 10 $\mu\text{l.}$ of a strong preparation of xanthopterin oxidase was added, and in 1.5 min. the fluorescence decreased to zero. (All values are corrected for blank.) Boiling for a short time in a free flame, followed by rapid cooling to room temperature, caused an increase in fluorescence to 19; upon addition of fresh enzyme the fluorescence once more dropped to zero. After this, no increase in fluorescence could be evoked by boiling.

Since the rate of conversion of X^0 to X is very slow at pH 7, almost no transformation could have taken place in the short time required for the oxidation of X . Upon boiling, the enzyme was irreversibly destroyed,

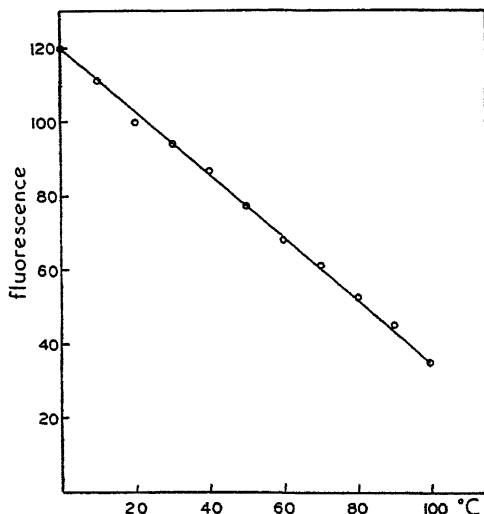


FIG. 4. Relation between fluorescence of xanthopterin and temperature of the solution. (200 $\mu\text{g.}$ of xanthopterin/ml. of 0.2 M secondary phosphate, pH about 9.) Abscissa: Temperature. Ordinate: Fluorescence.

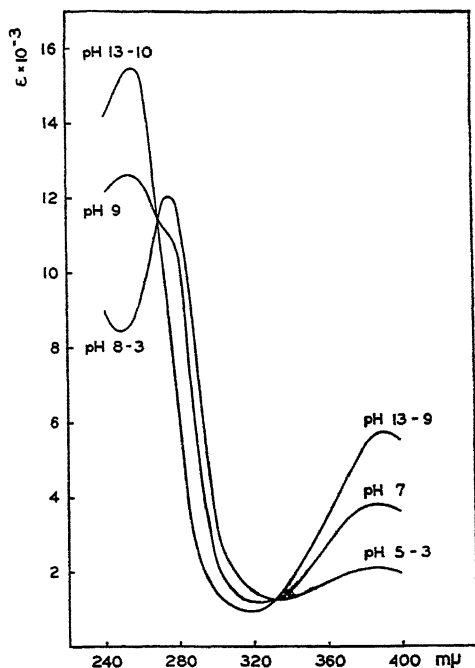


FIG. 5. Ultraviolet absorption spectra of xanthopterin at different hydrogen-ion concentrations. Abscissa: Wavelength. Ordinate: Molar extinction $\times 10^{-3}$

and the intact X^0 was quantitatively converted to fluorescent compound X . The newly formed X was then oxidized after addition of fresh xanthopterin oxidase.

From this experiment two more conclusions may be drawn. After the first addition of enzyme the fluorescence decreased to zero; this shows that X^0 is nonfluorescent. Furthermore, the increase in fluorescence upon heating (19 galvanometer divisions) was equal to the decrease brought about by a change from pH 13 to pH 7 ($100 - 80 = 20$). Hence, all of the xanthopterin must exist as X at pH 13. If one assumed that some X^0 was present at pH 13, the increase in fluorescence evoked by heating would have been greater than the decrease due to the change of pH. Therefore, any ordinate value in Fig. 3 represents the true percentage of X at the pH value concerned.

Ultraviolet Absorption of Xanthopterin

Investigations of the ultraviolet absorption of xanthopterin at different hydrogen-ion concentrations lead to conclusions closely corresponding to those drawn from the fluorescence measurements.

At all pH values above 3 xanthopterin absorbs ultraviolet irradiation; the absorption is strongest in the two regions, 255–275 $m\mu$ and ca. 390 $m\mu$ (Fig. 5). This is in agreement with most published data (6,8,23–25).

The absorption at the shorter wavelengths depends upon the hydrogen-ion concentration in the following way: From pH 13 to pH 10 the absorption maximum is at 255 $m\mu$, while from pH 8 to pH 3 it is located at 275 $m\mu$, and the extinction is considerably lower. Between pH 10 and pH 8 the curve takes an intermediate position. These variations of absorption with pH are instantaneous. In contrast, the changes occurring in the region 275–330 $m\mu$ are more sluggish; this corresponds to what is observed at 390 $m\mu$ (see below). The absorption at 330 $m\mu$ is the same at all hydrogen-ion concentrations between pH 13 and pH 3. At 390 $m\mu$ xanthopterin exhibits a second absorption peak, and the extinction of this maximum is also dependent upon the pH. In this region (as in the region 275–330 $m\mu$), phenomena very similar to those seen in the fluorescence measurements are observed (See Table III, cf. Table I.)

Here, also, more than one reaction is apparent: First, reaction *a*, an instantaneous decrease in optical density (at pH 7 from 0.650 to 0.553),

TABLE III

An alkaline solution of xanthopterin was diluted at zero time to a concentration of 20 $\mu\text{g./ml.}$ with buffer solution, and the optical density at 390 $m\mu$ was read at intervals.

Buffer solutions: 0.2 *M* phosphate buffer, pH 11.0; 0.2 *M* phosphate buffer, pH 7.0; 0.2 *M* phosphate buffer, pH 5.8; 0.2 *M* acetate buffer, pH 3.4.

| Time, min. | | 0 5 | 2 | 5 | 8 | 15 | 25 | 40 | 60 | 90 | 150 |
|---------------------------|------|-------------------------------|------|------|------|------|------|------|------|------|------|
| | pH | Optical density at 390 $m\mu$ | | | | | | | | | |
| pH changed from 13 to: | 11.0 | .650 | .649 | .648 | .649 | .649 | .652 | .649 | .647 | .654 | .653 |
| | 7.0 | .553 | .546 | .535 | .518 | .499 | .490 | .479 | .465 | .458 | .446 |
| | 5.8 | .442 | .405 | .340 | .311 | .286 | .280 | .272 | .267 | .268 | — |
| | 3.4 | .240 | .242 | .242 | .242 | — | — | — | — | .240 | — |

and then, reaction *b*, a slow decrease to a stable value (at pH 7 from 0.553 to 0.446). There is a corresponding two-step increase in absorption when the pH is shifted from pH 3 to around pH 7.

An experiment quite analogous to that shown in Fig. 1 was also performed (Table IV). By taking all readings immediately after adjusting the pH to 7.0, the effects of the rapid reaction *a* were eliminated, and it was possible to record only the progress of reaction *b* as the pH was shifted from 3 or 13 to intermediate values. In Table IV only the final, stable absorption values are listed and these were the same for a given pH, whether the initial xanthopterin solution was acid or alkaline. In this experiment, too, a minimum rate was observed at pH 7.0 (Fig. 2).

TABLE IV

Xanthopterin in 0.1 *N* sodium hydroxide or in 0.02 *M* acetate buffer, pH 3, was adjusted at zero time to different pH values by diluting in appropriate buffer solutions. At intervals samples were withdrawn, and the optical density at 390 m μ was read immediately after diluting to a concentration of 20 μ g./ml. with 0.2 *M* phosphate buffer of pH 7.0.

| Hydrogen-ion conc. brought from acid or alkaline to pH: | 4.1 | 5.0 | 6.4 | 7.0 | 7.7 | 9.0 | 10.3 | 11.0 |
|--|------|------|------|------|------|------|------|------|
| Optical density at 390 m μ after attainment of stable values | .295 | .294 | .341 | .445 | .510 | .520 | .514 | .545 |

These observations furnish further evidence of the existence of an equilibrium, $X \rightleftharpoons X^0$, as was inferred from the fluorometric data. But since the absorption of compound X^0 at 390 m μ is unknown the values in Table IV cannot be taken as a direct measure of the ratio, X/X^0 , at different hydrogen-ion concentrations.

In an attempt to determine the absorption spectrum of X^0 , advantage was taken of the fact that X^0 is not oxidized in the presence of xanthopterin oxidase. When xanthopterin, in the form of X , is enzymatically transformed to leucopterin, characteristic spectral changes are seen (22). There is an increase in optical density at 330 m μ and a decrease at 390 m μ . The decrease amounts to little more than half the increase at 330 m μ . When an enzyme preparation of sufficient activity is used, these changes are completed within a very short time, after which the reading remains constant.

This was shown by adjusting an alkaline solution of xanthopterin to pH 7.0 (pH optimum of the enzyme) with phosphate buffer and adding xanthopterin oxidase immediately. The stability in optical density after completed action of the enzyme gives additional evidence that xanthopterin exists wholly in the X form in alkaline solution.

If the enzyme, however, is added to a mixture of compounds X and X^0 , the changes in optical density at 330 $m\mu$ and 390 $m\mu$ take place in two steps: a rapid change, completed within a few minutes and due to the enzymatic transformation of compound X , and a slow change, lasting about 2 hr. and caused by the slow conversion of X^0 to X at pH 7, followed by the rapid oxidation of X to leucopterin (Fig. 6).

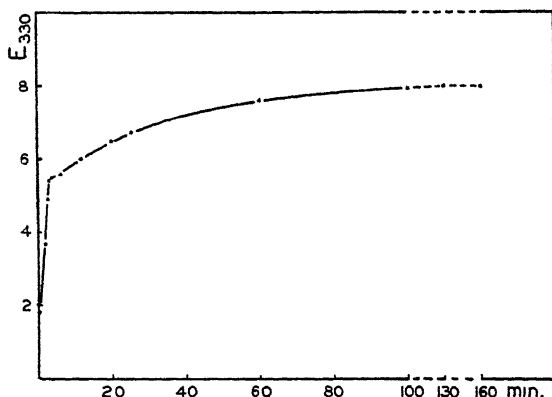


Fig. 6. Xanthopterin in 0.02 M acetate buffer, pH 3, was diluted at zero time to a concentration of 20 $\mu\text{g.}/\text{ml.}$ with 0.2 M phosphate buffer, pH 7.0. Fifty $\mu\text{l.}$ of xanthopterin oxidase was added immediately to each of the cells containing sample and blank (in order to correct for the ultraviolet absorption of the enzyme), and the optical density at 330 $m\mu$ was recorded at intervals. Abscissa: Time in minutes. Ordinate: Optical density at 330 $m\mu$.

This specificity of the enzyme makes it possible to determine the absorption spectrum of compound X^0 indirectly:

A.—At zero time xanthopterin in 0.1 N sodium hydroxide was diluted to a concentration of 20 $\mu\text{g.}/\text{ml.}$ with 0.2 M phosphate buffer, pH 7.0, and the absorption at different wavelengths in the region 240–400 $m\mu$ was measured at short time intervals. For each wavelength, the optical density was extrapolated back to zero time, and then plotted as spectrum A in Fig. 7.

B.—Xanthopterin in 0.02 M acetate buffer, pH 3, was diluted to a concentration of 20 $\mu\text{g.}/\text{ml.}$ with 0.2 M phosphate buffer, pH 7.0. The spectrum at zero time was computed and plotted as above. (Spectrum B, Fig. 7.)

C, D.—Xanthopterin in 0.02 *M* acetate buffer, pH 3, was diluted to a concentration of 20 $\mu\text{g./ml.}$ with 0.2 *M* phosphate buffer, pH 7.0. Fifty $\mu\text{l.}$ of xanthopterin oxidase was then immediately added to each of the cells containing sample and blank, and after a lapse of 3 min. the optical densities were measured at short time intervals until stable values were attained. The final spectrum is plotted in Fig. 7 as *C*. Spectrum *D* was obtained by extrapolation to zero time.

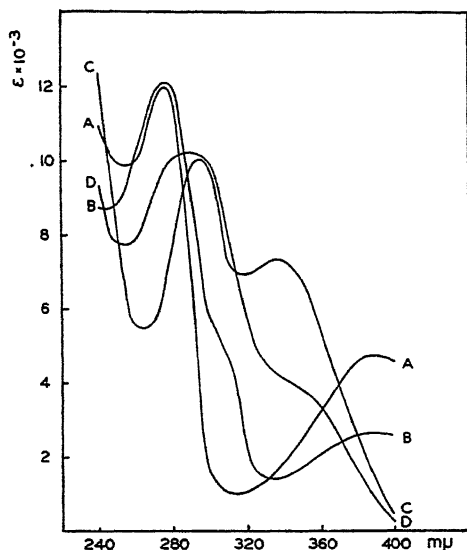


FIG. 7. *A*, spectrum of compound *X*; *B*, spectrum of *X* + *X*⁰; *C*, spectrum of leucopterin; *D*, spectrum of leucopterin + *X*⁰. All spectra were determined at pH 7.0. Abscissa: Wavelength. Ordinate: Molar extinction $\times 10^{-3}$.

The four spectra in Fig. 7 can now be used for calculating the percentage, *y*, of compound *X* present at pH 3. Spectrum *A* represents 100% of xanthopterin in the form of *X*, spectrum *B* the absorption of a mixture containing $\frac{y}{100} X + \frac{100-y}{100} X^0$. Likewise, *C* represents 100% leucopterin, and *D*, $\frac{y}{100}$ leucopterin + $\frac{100-y}{100} X^0$.

We now have:

$$\frac{100-y}{100} X^0 = B - \frac{y}{100} A = D - \frac{y}{100} C.$$

Hence,

$$y = \frac{(D - B) \times 100}{C - .1}.$$

This expression is valid for any wavelength in the region investigated, and the values of y thus obtained are essentially the same:

| | | | | | | | | | | | |
|---------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| m μ | 390 | 350 | 330 | 310 | 300 | 290 | 280 | 270 | 260 | 250 | 240 |
| y | 53 | 51 | 51 | 49 | 54 | 50 | 52 | 55 | 56 | 66 | 48 |
| Av. y | $= 53\%$ | | | | | | | | | | |

Once y is known, we are able to calculate the absorption spectrum of xanthopterin wholly in the form of X^0 by either of two equations:

$$X^0 = \frac{100}{47} \times (B - 0.53 \times A), \quad [1]$$

or

$$X^0 = \frac{100}{47} \times (D - 0.53 \times C). \quad [2]$$

Fig. 8 shows the spectrum as calculated for the region from 240 to 400 m μ . It can be seen that the conversion of compound X to compound X^0 is accompanied by a disappearance of the absorption peak at 390 m μ and the emergence of a "swelling" at about 300 m μ .

From Fig. 8 we are now able to read the extinction of X^0 at 390 m μ ($\epsilon \times 10^{-3} = 0.3$), and this permits us to calculate the percentages of X at any hydrogen-ion concentration between pH 13 and 3 from the data in Table IV. The calculated percentages are plotted against pH in Fig. 3, and they can be seen to correspond rather closely to the percentages obtained from the fluorometric data.

Example of calculation:

At 390 m μ 100% X gives an $\epsilon \times 10^{-3}$ of 4.8 and 100% X^0 an $\epsilon \times 10^{-3}$ of 0.3. At pH 7.0 the final absorption value is 0.445, which corresponds to an $\epsilon \times 10^{-3}$ of 4.0. This gives a percentage of compound X at pH 7.0 equal to: $\frac{(4.0 - 0.3) \times 100}{4.8 - 0.3} = 82\%$.

Measurements of the absorption at 390 m μ confirm the conclusions drawn from the fluorometric data as to the influence of cooling and heating on the ratio X/X^0 and on the speed with which equilibrium is attained.

Keto-Enol Tautomerism of Xanthopterin

In the preceding sections it has been shown that xanthopterin may undergo a partial and reversible conversion to another compound, X^0 , which differs from xanthopterin, X , in optical properties and in susceptibility to xanthopterin oxidase. It will appear from the arguments to follow that this conversion probably is a tautomeric shift from an enol to a keto form at carbon atoms 6 and 7.

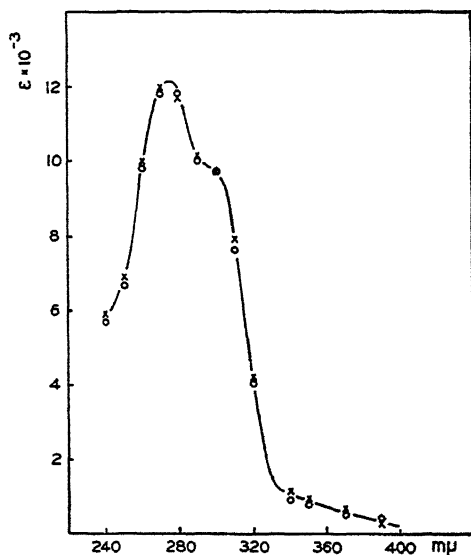
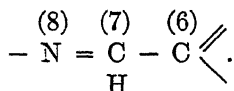


FIG. 8. Ultraviolet absorption spectrum of compound X^0 at pH 7.0. O, calculated from Eq. [1]; X, calculated from Eq. [2]. Abscissa: Wavelength. Ordinate: Molar extinction $\times 10^{-3}$.

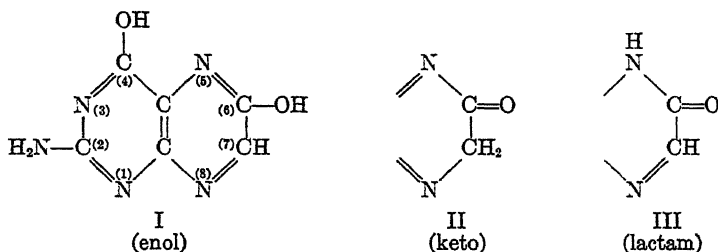
It is known that xanthopterin oxidase catalyzes an oxidation at carbon atom 7 in the pyrazine ring of some pterines, and since compound X^0 is not oxidized when enzyme is added, the change in configuration from X to X^0 must be supposed to take place in this ring. This is indirectly supported by the observation (21) that 2,4,6-trihydroxypteridine (desiminoxanthopterin) is also oxidized in the presence of xanthopterin oxidase. This indicates that a change in the substituents of the pyrimidine ring does not alter the susceptibility to the enzyme.

Furthermore, 2-amino-4-hydroxypteridine (AHP), which differs from xanthopterin only in regard to a hydroxyl group in the pyrazine ring, does *not* show such changes in optical properties and oxidizability as does xanthopterin.

As shown by Lowry *et al.* (20), AHP is oxidized in the presence of xanthopterin oxidase to 2-amino-4,7-dihydroxypteridine (isoxanthopterin). This means that an oxidation at carbon atom 7 is possible when the configuration in the pyrazine ring is as follows:



The difference in oxidizability of the compounds X and X^0 might now be explained by assuming that in X the 6-hydroxy group is in the enol form (formula I) which contains carbon atom 7 in a linkage similar to that mentioned above, and that in X^0 the corresponding keto form (formula II) is present. For compound X another tautomeric structure, the lactam form (formula III), is possible, but the absorption spectrum of X strongly indicates that it exists predominantly in the enol (lactim) form. Furthermore, Purrmann (26) concluded from a comparison of the acidic properties of xanthopterin and isoxanthopterin that the pyrazine ring of the former is in the lactim form.



The assumption of a keto-enol tautomerism would be consistent with the observation (Fig. 2) that the attainment of the equilibrium is acid-base catalyzed, as this form of catalysis is known to characterize prototropic isomerizations.

During the conversion of X to X^0 , the ultraviolet maximum is shifted towards shorter wavelengths. This gives further support to the hypothesis advanced. According to modern theories of light absorption, the maximum would move in the direction indicated when the extended

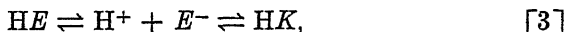
conjugation of the double bonds prevailing in the enol form is restricted to a smaller part of the molecule as is the case in the keto tautomer. Similar changes in the absorption spectrum of xanthopterin are observed when the number of conjugated double bonds is decreased by hydrogenation as in the dihydroxanthopterins of Hitchings and Elion (27) and O'Dell *et al.* (25).

Finally, it has been possible to show that a solution of xanthopterin wholly in the form of compound *X* has a higher content of phenolic hydroxyl groups than does a mixture of *X* and *X*⁰. When excess ferric chloride is added to xanthopterin, a complex iron salt with maximum absorption at 450 mμ is formed. Presumably the extinction at this wavelength can not be taken as a quantitative measure of the amount of enolic tautomer present, especially since nothing is known of the tautomeric form of the 4-hydroxy group in the pyrimidine ring. However, the difference revealed in the following experiment is sufficiently great to support the assumption of the enol configuration for compound *X*.

An alkaline solution of xanthopterin was diluted to a concentration of 30 μg./ml. with 0.2 *M* maleate buffer at pH 7.0 and kept at room temperature for 2 hr. to reach equilibrium. An aliquot of this solution was heated to 100°C. for 2 min. and then rapidly cooled to room temperature. To each of the cells containing sample and blank, 25 μl. of a solution containing 100 mg. ferric chloride/ml. water was immediately added. This procedure caused an increase in optical density at 450 mμ from 0.053 to 0.271; *increase: 0.218*.

A second aliquot was cooled to -12°C. for 30 min. and after rapid heating to room temperature ferric chloride was added as above. In this case the optical density at 450 mμ rose from 0.052 to 0.202; *increase: 0.150*.

As can be seen from Fig. 3, the curve has a striking resemblance to an acid dissociation curve with p*K* at about 7. An obvious conjecture would therefore be that the transformation in question is actually an enol-enolate-keto equilibrium of the form:



where *HE* is the undissociated enol, *E*⁻ the conjugated base (an enolate ion) and *HK* the keto form.² The rate of appearance and disappearance of *HK* must then be dependent upon the pH as shown in Fig. 2, while the dissociation of *HE* is probably an instantaneous reaction (Fig. 9).

² Another possibility is: $HK \rightleftharpoons HE \rightleftharpoons H^+ + E^-$. It is at present not possible to determine which expression is the correct one.

In Fig. 3 there was plotted a single value obtained after "incubating" xanthopterin at pH 1.3. The position of this reading could indicate the appearance of an additional compound and one might hypothesize the formation of an "enolonium ion", H_2E^+ , but the behavior of xanthopterin in this region has not been more closely investigated.

If the conversions are, in fact, acid-base equilibria of the form given in Eq. [3], it should be possible to obtain information about the true charges of the molecules involved by observing the change in the equilibrium resulting from a change in ionic strength of the medium (Table V). These experiments were all performed at a pH value around 7 but in two buffer systems of different valence type. The ionic strength was altered by the addition of sodium chloride in varying amounts.

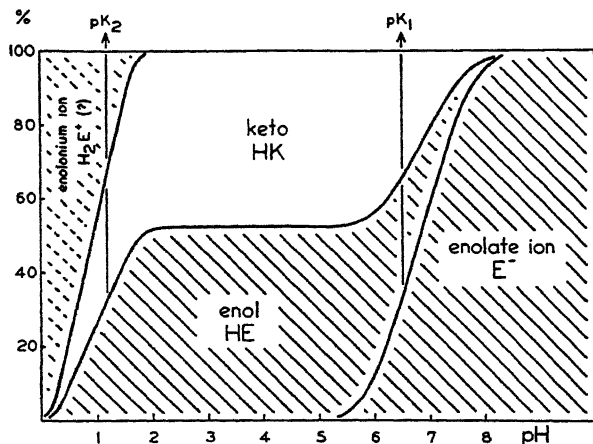
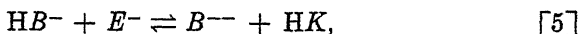
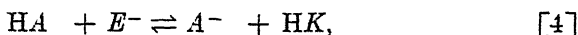


FIG. 9. Diagram of the enolate-enol-keto(-enolonium?) equilibrium at different hydrogen-ion concentrations. The cross-hatched areas represent fluorescent compounds. Abscissa: pH. Ordinate: Per cent of total xanthopterin.

The equilibria investigated in these experiments are, in fact, the following:



where HA/A^- represents the dimethylbarbiturate buffer system and HB^-/B^{--} the phosphate buffer system.

If the charges of keto, enol, and enolate ion, actually, are as those given in Eq. [3], the addition of neutral salt in the first part of the experiment (Eq. [4]) should not be expected to cause any change in the amount of HK as in this case the activity coefficients will be influenced to the same extent on both sides. (Σz^2 is equal to $1^2 = 1$ on both sides; z is the charge on the ions concerned.)

On the other hand, in the second part of the experiment (Eq. [5]), Σz^2 on the right side is equal to $2^2 = 4$, while $\Sigma z^2 = 1^2 + 1^2 = 2$ on the left. Hence, a salt effect, displacing the equilibrium in favor of the uncharged keto form, HK, should be apparent in this case. This is actually borne out by the results in Table V.

TABLE V

The percentage of the keto tautomer (compound X⁰) at pH 7 was determined as follows:

Alkaline solutions of xanthopterin were adjusted to pH 11 and pH 7, respectively, by diluting to a concentration of 10 $\mu\text{g.}/\text{ml.}$ with the appropriate buffer solutions. After 2 hr. at room temperature both samples were diluted to a final concentration of 100 $\text{m}\mu\text{g.}/\text{ml.}$ with buffer solution of pH 7, and the fluorescence was read immediately.

Calculation:

$$\frac{(\text{flu. of sample kept at pH 11} - \text{flu. of sample kept at pH 7}) \times 100}{\text{flu. of sample kept at pH 11}} = \text{per cent keto.}$$

(All readings corrected for blank.)

| Ionic strength (molarity) | 0.01 M Dimethylbarbiturate buffer + sodium chloride | | | 0.01 M Phosphate buffer + sodium chloride | | |
|---------------------------|---|-----|-----|---|-----|-----|
| | 0.02 | 0.1 | 0.5 | 0.02 | 0.1 | 0.5 |
| Per cent keto at pH 7 | 20 | 23 | 21 | 16 | 23 | 28 |

So it seems justified to conclude that it is the first acid dissociation constant of xanthopterin that has been determined. Fig. 9 reveals that at 20–22°C. the pK_1 in 0.2 M phosphate buffer must be about 6.5 for the enol as well as the keto form since at $\text{pH} \approx 6.5$ we have: $C_{\text{HE}} \approx C_{\text{E}^-} \approx C_{\text{HK}}$.

Since the ratio HE/HK is almost equal to unity at room temperature, the acid dissociation constants and hence the free energies of dissociation of the two tautomers must be practically identical. They must, however, differ with regard to heat of dissociation as is revealed by the change in ratio brought about by a change of temperature.

Lowry *et al.* (20) pointed out that the fluorescence of xanthopterin is influenced by ions differently below and above pH 8.5. At lower pH values a number of anions exert a depressing ("quenching") effect on the fluorescence while in more alkaline solution the fluorescence is increased by di- and trivalent cations. A possible explanation of these phenomena might be that the fluorescence of the undissociated enol and the enolate ion differ in sensitivity toward the ions mentioned. It is also possible that the *rapid* changes in ultraviolet absorption seen after a change in the hydrogen-ion concentration might be due to differences in light absorption by the different ionic states of xanthopterin. These problems will have to be solved by further research.

ACKNOWLEDGMENTS

I want to express my gratitude to the head of this institute, Dr. Herman M. Kalckar, and to Dr. Jørgen Koefoed, the Physicochemical Institute, University of Copenhagen, for help and advice.

SUMMARY

It has been shown that a change in hydrogen-ion concentration brings about reversible changes in the optical properties and the susceptibility to enzymatic oxidation of xanthopterin.

The phenomena observed can be explained by assuming a reversible keto-enol transformation to take place at carbon atoms 6 and 7. The first acid dissociation constant of xanthopterin is about 6.5. At higher pH values xanthopterin will exist mainly as the enolate ion. In more acid solution at room temperature almost equal amounts of the undissociated enol and keto tautomers will be present. The ratio of enol to keto form will change when the temperature is changed.

The keto form of xanthopterin is nonfluorescent; it is not oxidized in the presence of xanthopterin oxidase; its dissociation, as well as its isomerization to the enol form, is a relatively slow process. The enol form dissociates instantaneously.

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Dismutative Assimilation of Carbon Dioxide

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INTRODUCTION

Micrococcus pyogenes var. *aureus* effects the anaerobic dismutation of pyruvic acid in which one molecule is oxidatively decarboxylated to acetic acid and a second is reduced to lactic acid:



The reaction is frequently found to occur in bacterial dissimilation; however, if the reaction is reversible, it would constitute an assimilation of CO_2 .

It has been found that the dismutation reaction is reversible with respect to lactate and carbon dioxide, but that acetate as such does not participate in the reversibility. Thus an additional type of CO_2 fixation is shown to occur in bacterial metabolism, an assimilation which involves $\text{CO}_2 + \text{C}_2(4,5)$.

METHODS

Nonproliferating cells or cell-free extracts were used. *Micrococcus pyogenes* var. *aureus* was grown aerobically at pH 7.4 on a medium containing 1.0% peptone, 0.5% yeast extract, 0.5% beef extract, 0.5% medium chloride, and 2.0% agar. The cells were harvested and lyophilized after incubating 20–24 hr. at 30°C. Cell-free extracts were prepared by grinding freshly harvested cells with powdered glass (7).

Reversibility was determined by incubating pyruvate with cells or cell-free extracts at pH 6.6–6.8 in the presence of isotopically-labeled bicarbonate, acetate, or lactate. Metabolic reactions were carried out on a Barcroft-Warburg respirometer at 30.3°C. Anaerobiosis was maintained with oxygen-free nitrogen which had been passed over hot copper turnings. In certain experiments, yellow phosphorus was used in the center well of the flask to insure complete anaerobiosis.

Isotopic $\text{NaH}^{13}\text{CO}_3$ was prepared by absorbing C^{13}O_2 in a solution containing an equivalent amount of sodium hydroxide. Carboxyl-labeled lactate was prepared by a

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modification of the method described by Sakami *et al.* (8). Carboxyl-labeled acetate was prepared from $C^{13}O_2$ by the Grignard reaction.²

Activity of the enzymes was stopped when the reaction was one-third to one-half complete by adding sulfuric acid. Pyruvic acid was separated from lactic and acetic acids by extracting with ether continuously for 15 hr. in the presence of 5% sodium bisulfite. Lactic and acetic acids were separated by triple steam distillation. Acetic acid was oxidized to carbon dioxide and water by wet combustion with potassium persulfate (9). Lactic acid was decarboxylated with permanganate (10) to form CO_2 and acetaldehyde which was further degraded by the iodoform reaction. Iodoform, representing the methyl group of lactic acid, was converted to CO_2 by wet combustion. Formic acid, representing the α -carbon of lactic acid was distilled and oxidized to CO_2 with mercuric oxide (11).

Unless indicated otherwise, pyruvic acid was degraded by a cell-free extract from *Aerobacter aerogenes*. Acetylmethylcarbinol, representing the methyl and carbonyl carbons of pyruvate, and carbon dioxide, representing the carboxyl carbon, are formed by this preparation. Oxidation of acetylmethylcarbinol with periodic acid yields acetic acid and acetaldehyde (12). The products of this oxidation were converted into carbon dioxide by methods described above.

In all cases, carbon dioxide was absorbed in 4 *N* CO_2 -free alkali and precipitated as barium carbonate by adding a saturated solution of barium nitrate. The samples of barium carbonate were washed thoroughly, dried at 110°C. and preserved in stoppered vials. The C^{13} content was determined by means of a Nier mass spectrometer. Isotope concentrations greater than 0.05 atom-% excess are considered significant.

EXPERIMENTAL

Reversibility With Respect to Lactate

Carboxyl-labeled lactate should yield an excess of the isotope in carbon dioxide as well as in the carboxyl of pyruvate if the reaction is reversible. Results (Table I) show an excess of C^{13} in carbon dioxide and in the pyruvate carboxyl thus indicating reversibility with respect to lactate. The value of 0.15 atom-% excess of the α -carbon of lactate (col. 5, Table I) is believed to be a result of contamination since other experiments showed no excess C^{13} in this position.

As a result of technical difficulties encountered in obtaining clear-cut separation of the products, their quantitative determinations are only approximations. Carbon balances show 98.5% total recovery. Lactate is readily assimilated during the reaction since there is no accumulation above the amount initially added. The disappearance of lactate in the experiment containing only labeled lactate indicates that this compound is consumed anaerobically by another process. The isotope dilution is

² Appreciation is expressed to W. B. Sutton for synthesizing the labeled acetate.

TABLE I

Reversibility of Dismutation of Pyruvate With C¹³ Lactate

Each 125-ml. Warburg cup contained 450 mg. lyophilized cells of *M. pyogenes* var. *aureus*; 1.50 mmoles potassium phosphate buffer pH 6.4; 4 ml. 12 *N* sulfuric acid in side arm and the additions stated. Yellow phosphorus in center well. Gassed with oxygen-free nitrogen.

| Product | Endogenous | | C ¹³ lactate C ¹² excess | C ¹³ lactate + pyruvate | |
|-----------------|------------------------------|------------------------------|---|------------------------------------|-------------------------------------|
| | | | | | C ¹² excess ^b |
| Carbon dioxide | mmoles ^a 0.002 | mmoles ^a 0.004 | atom-% ^b 0.07 | mmoles ^a 0.371 | atom-% 0.14 |
| Lactate: | 0.008 | 0.206 | — | 0.347 | — |
| Carboxyl carbon | — | — | 5.35 | — | 1.61 |
| α-Carbon | — | — | -0.04 | — | 0.15 |
| Methyl carbon | — | — | 0.03 | — | 0.01 |
| Pyruvate: | 0 | 0 | — | 1.48 | — |
| Carboxyl | — | — | — | — | 0.50 |
| Additions: | | | | | |
| Lactate | 0 | 0.40 | 7.25 | 0.40 | 7.25 |
| Pyruvate | 0 | 0 | — | 1.83 | — |

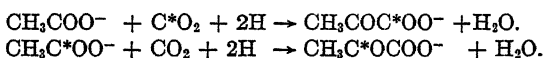
^a The analytical results recorded are approximations only.

^b Atom-% excess = $\left(\frac{C^{13}/C^{12}}{1 + C^{13}/C^{12}} \times 100 \right)$.

greater than can be accounted for by metabolic lactate, again indicating another pathway by which lactate is metabolized. The nature of this process was not investigated.

Reversibility With Labeled Bicarbonate and Acetate

Exchange of isotopically-labeled bicarbonate and carboxyl acetate should result in an excess of isotope in the carboxyl carbon and carbonyl carbon of pyruvate, respectively:



A hypothetical half a molecule of oxygen would be formed by the addition of carbon dioxide to acetate. Experiments were conducted, therefore, to test the exchange of isotopic carbon and hence reversibility

TABLE II

*Exchange of C¹³ of NaHC¹³O₃ with Pyruvate Carboxyl in Dismutation
Reaction With M. pyogenes var. aureus*

Each cup contained 1.30 mmoles pyruvate; 1.5 mmoles phosphate buffer at pH 6.6 plus additions listed.

| Addition | Pyruvate remaining mmoles | C ¹³ excess in carboxyl atom-% ^c |
|---|---------------------------------|--|
| Cells | | |
| 450 mg. lyophilized cells; 1.42 mmoles NaHC ¹³ O ₃ , (3.29 Atom-% excess) | 0.478 | 0.00 |
| Above additions plus 20 mg. ATP [Pyruvate decarboxylated by yeast carboxylase] | 0.351 | 0.07 |
| Cell-free extract | | |
| 15 ml. extract; 1.8 mmoles NaHC ¹³ O ₃ , (5.0 Atom-% excess) | 0.398 | 0.06 |
| Above additions plus 20 mg. ATP [(Pyruvate decarboxylated by Ce (SO ₄) ₂)] | 0.334 | 0.19 |

TABLE III

*Reversibility of the Dismutation Reaction With
Carboxyl-Labeled Acetate*

Each cup contained 450 mg. cells of *M. pyogenes* var. *aureus*; buffer pH 6.4; 2.0 ml. 12 N H₂SO₄; total volume 30 ml. Yellow phosphorus in center well. Gassed with oxygen-free nitrogen.

| Product | C ¹³ acetate + pyruvate | | C ¹³ acetate + pyruvate + 20 mg. ATP | |
|-----------------------|------------------------------------|------------------------|--|------------------------|
| | | C ¹³ excess | | C ¹³ excess |
| | mmoles | atom-% | mmoles | atom-% |
| CO ₂ | 0.34 | 0.03 | 0.40 | 0.04 |
| Pyruvate: | 0.36 | | 0.33 | |
| Carboxyl | | 0.02 | | 0.06 |
| Carbonyl | | 0.02 | | 0.02 |
| Methyl | | -0.01 | | -0.03 |
| Acetate: | | | | |
| Carboxyl ^a | 1.43 | 0.17 | 1.62 | 0.70 |
| | | 0.34 | | 1.40 |
| Additions: | | | | |
| Pyruvate | 1.75 | | 1.75 | |
| Acetate | 0.40 | 2.91 ^b | 0.40 | 2.91 |

^a Calculated.

^b C¹³ content of carboxyl carbon.

with respect to C_1 and C_2 addition. Results (Table II) obtained by adding labeled carbon dioxide in the form of sodium bicarbonate to the metabolizing system show that an exchange does take place with the carboxyl carbon of pyruvate. Adenosine triphosphate (ATP) enhances the exchange and hence the fixation of CO_2 in both cells and in cell-free extracts. Dismutation of pyruvate by *M. pyogenes* var. *aureus*, therefore, is reversible with respect to carbon dioxide.

Experiments (Table III) in which carboxyl-labeled acetate was used indicate, however, that acetate as such is not a participant in the reverse reaction. An excess of isotope should appear in the carbonyl carbon of pyruvate. The results show that a significant excess of C^{13} was not in this position. Adenosine triphosphate shows no observable effect in enhancing the exchange from acetate to pyruvate. In one case a questionably significant excess (0.06 atom-%) in the carbonyl position of pyruvate (col. 4) could be not repeated. The results in Table IV were obtained with cells; studies with cell-free extracts showed similar results.

DISCUSSION

Exchange of labeled lactate with pyruvate is to be expected if, as formulated by Lipmann (13), lactic dehydrogenase functions in dismutation. Lactic dehydrogenase catalyzes reversibly the removal of hydrogen from lactate thereby forming pyruvate. No difficulty should be experienced, therefore, in obtaining an excess of isotope in the carboxyl position of pyruvate, and hence in metabolic carbon dioxide.

Failure of acetate to participate in the reverse reaction is in accordance with results of studies of the phosphoroclastic reaction by Strecker *et al.* (14), although earlier studies of the latter reaction indicated that acetate did participate in $CO_2 + C_2$ addition to form pyruvate (4,5). Some C_2 fragment other than acetate must be the active participant. A likely intermediate is acetyl phosphate in which case adenosine triphosphate should enhance the exchange with labeled acetate. Adenosine triphosphate did not enhance the reversibility of acetate in the dismutation reaction suggesting that acetyl phosphate is not the intermediate or that the preparation used did not have the enzyme system for phosphorylating acetate. In view of the results of Strecker *et al.* and of the authors (15) in which formation of acetyl phosphate could not be detected during dismutation by *M. pyogenes* var. *aureus*, one would conclude that acetyl phosphate is not the direct intermediate.

The role of adenosine triphosphate in enhancing the reversibility of the dismutation is not clear. That adenosine triphosphate enhances the fixation of carbon dioxide yet has no effect on acetate indicates that the energy required for $\text{CO}_2 + \text{C}_2$ addition is associated with carbon dioxide. The energy-rich pyrophosphate linkage of ATP may be utilized in activating carbon dioxide through phosphorylation of bicarbonate, perhaps, thereby paving the way for the addition reaction. Such an explanation, at present, has no experimental proof.

SUMMARY

1. The dismutation reaction carried out by cells and cell-free extracts of *Micrococcus pyogenes* var. *aureus* is reversible with respect to lactate and carbonate. Acetate, as such, does not participate in the reverse reaction.

2. Reversibility of the dismutation reaction represents another pathway by which carbon dioxide may be fixed and assimilated. Its importance is not known as yet.

3. Adenosine triphosphate enhances the fixation of carbon dioxide by dismutation yet has no effect on the exchange of acetate.

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A Rapid Method for the Estimation of the Glutamic-Aspartic Transaminase in Tissues and Its Application to Radiation Sickness

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INTRODUCTION

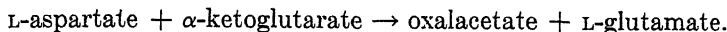
A convenient method for the estimation of the transaminase content of tissues would permit the study of a variety of problems which are at present difficult to approach. The established relationship between vitamin B₆ and transamination (1,2,3) renders such determinations especially pertinent. The results of previous estimations of transaminase activity in tissues have been reviewed elsewhere (4,5). Three methods are employed at present: that of Cohen (6) using chloramine-T, that of Cohen and Hekhuis (7) based on more extensive analysis, and that of Ames and Elvehjem (8,9) based on the methods devised by Green *et al.* (1). These require manometric equipment, are not suited to large scale or routine application, and in various other ways leave much to be desired. We have now devised a simple colorimetric method for transaminase. It is based upon the conversion of aspartic acid to oxalacetic acid by the enzyme, the chemical conversion of the oxalacetate formed to pyruvate, and the colorimetric estimation of the latter. Manipulation is at a minimum and relatively accurate results are obtained rapidly. The method is applied to the determination of the transaminase activities of tissues of normal mice and of mice exposed to sublethal doses of radiation. This experiment logically follows the report of Goldfeder *et al.* (10) that increased vitamin B₆ in the diet permitted better survival in irradiated animals. It seeks to answer the question as to whether the transaminase activity of irradiated animals is decreased.

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METHOD FOR ESTIMATING TRANSAMINASE

Principle

The reaction studied in all cases reported here is the following:



This reaction is allowed to proceed for a definite time in the presence of excess aspartate and α -ketoglutarate, so that less than 5% of the α -ketoglutarate is transaminated. The reaction is stopped and the oxalacetate converted to pyruvate by means of aniline citrate. The pyruvate is then determined, in the presence of excess α -ketoglutarate, by a simple modification of the Friedemann-Haugen method (11).

Reagents

Buffered aspartate. DL-Aspartic acid (1.33 g.) and 0.86 g. K_2HPO_4 are dissolved in water, adjusted with KOH to pH 7.4 and diluted to 50 ml. The water employed in this and other reagents was redistilled from an all-glass apparatus.

α -Ketoglutarate. One-tenth molar, adjusted to pH 7.4 with KOH.

Aniline citrate. Aniline; a citric acid solution made by dissolving 50 g. citric acid in 50 ml. water. Equal parts of citric acid and aniline are mixed just before use.

Buffer. One-tenth molar potassium phosphate, pH 7.4.

Trichloroacetic acid. One hundred grams of trichloroacetic acid made to 100 ml. with water.

Dinitrophenylhydrazine reagent. One hundred milligrams of 2,4-dinitrophenylhydrazine dissolved in 20 ml. concentrated HCl and 80 ml. water.

Toluene. Water saturated. Reagent-grade toluene did not require purification.

Alcoholic KOH. KOH, 2.5 g. made up to 100 ml. with 95% alcohol.

METHOD

A series of small test tubes, rinsed with glass-distilled water, are placed in a water bath at 37°C. The tissue to be assayed is added. In the case of rat or mouse kidney, liver, heart, and brain we have added 0.05 or 0.1 ml. of a 1% homogenate (1 ml. equivalent to 10 mg. wet-wt. tissue) made in ice-cold buffer prepared with glass-distilled water. Coenzyme preparations or other materials are added at this time. We have held the total volume of tissue plus other additions to 0.2 ml. and any volume less than this was made to 0.2 ml. by the addition of buffer. To each tube was then added 0.5 ml. of the buffered aspartate. After the mixture had reached the temperature of the bath (4-5 min.), 0.2 ml. of α -ketoglutarate at 37°C. was added, with mixing. The mixture was incubated for exactly 10 min., after which 0.1 ml. of trichloroacetic acid was added and the tubes shaken to precipitate the proteins and stop the reaction. At some convenient time thereafter, 0.1 ml. of aniline citrate was added, the tubes were shaken to disperse the reagent and allowed to incubate for approximately 10 min. The time is not critical and the procedure may be interrupted for hours at this point. The

keto acids in the tube now constitute the excess α -ketoglutarate added, and the pyruvate derived from the oxalacetate formed by the reaction.

To each reaction tube, without the necessity of removing the protein precipitate, was added 1 ml. of the 2,4-dinitrophenylhydrazine reagent, and the reaction was allowed to proceed (at 37°C.) for 5 min. Time is somewhat critical at this point but a period of 4-6 min. appeared to be quite satisfactory. Two ml. of water-saturated

TABLE I
Typical Data Derived from Transaminase Method Described

Part A: Pyruvate Recovery From Heart Tissue^a

| Pyruvate added μ g. | Colorimeter reading | Pyruvate recovered μ g. |
|----------------------------|---------------------|--------------------------------|
| 000 | 0 | 0 |
| 20 | 80 | 21 |
| 40 | 148 | 39 |
| 60 | 220 | 58 |
| 100 | 348 | 80 |

Part B: Relation Between Tissue Level and Activity (Mouse Liver)^b

| Wet-wt. tissue mg. | System | Colorimeter reading |
|-----------------------|---------------------------------|---------------------|
| 0.5 | Complete | 80 |
| 1.0 | Complete | 165 |
| 1.5 | Complete | 240 |
| 2.0 | Complete | 360 |
| 1.0 | Without α -ketoglutarate | 17 |
| 1.0 | Without aspartate | 0 |
| 1.0 | Without both | 20 |
| 0.5-2.0 | Complete, zero time | 0 |

Part C: Replication of Samples (Mouse Heart)

| Wet-wt. tissue mg. | Colorimeter reading |
|-----------------------|---------------------|
| 1.0 | 236 |
| 1.0 | 224 |
| 1.0 | 225 |
| 1.0 | 214 |

^a Pyruvate was added to 1 mg. wet-wt. mouse heart and incubated for 10 min. in the usual method except that aspartate and α -ketoglutarate were omitted. Except at relatively high levels of pyruvate essentially none disappears under these conditions.

^b The dry weight of this particular liver was 27.1%, thus 1 mg. wet tissue = 0.271 mg. dry tissue. In 10 min., 1 mg. of wet tissue produced 45 μ g. of pyruvate. Since 1 μ mole of pyruvate = 22.4 μ l. = 88 μ g., the 45 μ g. pyruvate found is equivalent to 11.4 μ l. per hour, therefore, $11.4 \times 6 = 68.4 \mu$ l. of pyruvate would have been formed had the reaction continued at the same rate. Thus the Q_P^{10} would be:

$$Q_P^{10} = \frac{11.4 \times 6}{0.271} = 252.$$

toluene was then added to each tube and the whole shaken vigorously. The tubes were allowed to stand for about 5 min. during which time the toluene formed a clear layer at the top. If emulsions form they are broken by centrifugation. One ml. of the toluene layer was removed to a colorimeter tube containing 5 ml. of alcoholic KOH. The toluene was mixed with the KOH and the tube allowed to stand for 5-10 min. At the end of this time, 1 ml. of distilled water was added to dissolve any turbidity due to K_2CO_3 which may have precipitated and the color intensity was then read in a suitable instrument. We have used a Klett-Summerson photoelectric colorimeter with the green filter.

The color is standardized against redistilled pyruvate or purified sodium pyruvate, suitable aliquots of a known solution being added to the inactivated enzyme system and carried through the above procedure. So long as less than 5% of the α -ketoglutarate present has been transaminated the method fulfills the usual criteria of adequacy: straight line response to increasing concentration of enzyme, complete recovery of pyruvate or oxalacetate added to tissues in the absence of amino acid additions, and essentially identical colorimeter readings on replicate determinations. Data illustrating these points and showing methods of calculation are given in Table I. In our hands, pyruvate found (or total keto acid apparent by this method) in enzyme samples or tissue homogenates inactivated at zero time was negligible. However, in any tissue one would include two types of controls: one stopped with trichloroacetic acid at zero time, the other incubated for 10 min. without the addition of the α -ketoglutarate. However, in the tissues we have examined, hardly measurable quantities of pyruvate were found in either control. The method is thus simple and convenient and would seem to be an adequate measure of transaminase. The strictest test of any such method is in its routine use. We therefore present an experiment on radiation sickness which illustrates the use of the method and has in addition some intrinsic value of its own.

AN EXPERIMENT ON RADIATION SICKNESS

There are several indications in the literature that supplementary vitamin B_6 is helpful in combatting the after effects of excessive exposure to ionizing radiation. While the reproducibility is somewhat less than one would like, it must be recognized that radiation sickness is a complex phenomenon and that a great many of the variables involved are not known. Nevertheless, the observations raise the question of whether the activity of the enzymes with which vitamin B_6 is associated are impaired in the diseased animal.

To investigate this question, 41 white mice were chosen at random from the stock colony and placed on a normal mixed diet. Transaminase was determined on the heart and liver of five animals. Four days thereafter the remaining mice were transported to the Radiological Research Laboratory of the College of Physicians and Surgeons, Columbia University. The radiation was administered under the direction of Dr. G. Failla to whom we are indebted for his coöperation throughout

this experiment. One half of the animals, selected at random, were irradiated, six at a time. Each mouse received a whole body dose of 530 r of filtered 185 Kup x-rays, at the rate of 53 r/min. for a period of 10 min. The mice were held in a circular Plexiglas container in a fixed position during irradiation. That the dose was adequate is evident from the fact that all of the irradiated animals later developed radiation sickness. After exposure, the animals were marked and returned to the original colony so that irradiated and control animals were intermingled and living in the same cage. The entire lot was then transported back to our own laboratories where they were maintained on a

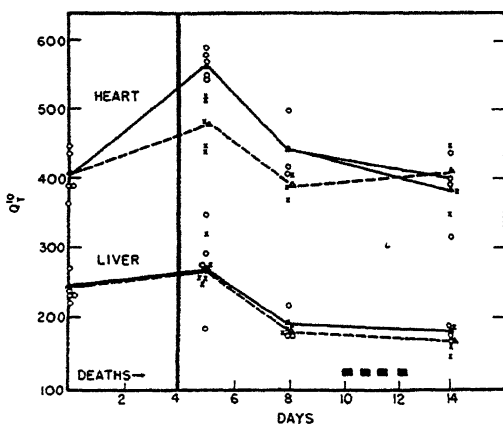


Fig. 1. Transaminase activity in tissues from irradiated and normal animals. Straight lines = normal animals. Dashed lines = irradiated animals.

normal mixed diet. One day after irradiation 5 animals from each group were analyzed. It was necessary at this point to separate the two groups since the irradiated animals became sick and were unable to obtain adequate food in competition with the controls. The groups were therefore kept separate for a period of 12 days by which time the irradiated animals had either recovered, died, or been sacrificed. At this time, all the remaining animals were again placed in the same cage. One animal in the irradiated group survived after a period of illness and 2 months later was apparently perfectly normal. All of the rest either died of the disease or were sacrificed.

For the estimation of transaminase the animal was killed by a blow on the head, and the tissue (heart and liver) removed to ice-cold buffer.

Immediately thereafter the tissue was blotted dry, weighed, and divided into two weighed portions. One of these was dried at 110°C. for 1 hr. and placed in a vacuum desiccator containing Drierite for 24 hr. for a determination of dry weight. The other portion was placed in an homogenizing tube, with 100 times its weight of ice-cold buffer (thus yielding a 1.0% homogenate), and homogenized in the cold. It was found possible to hold such homogenates in the cold for as long as 6 hr. without detectable loss of transaminase activity. Therefore a series of homogenates was collected and the transaminase activities determined as described previously. All transaminase determinations were made in duplicate at two levels of tissue. At each sampling the animals were chosen purely at random, and irradiated and control animals were intermingled. Heart was chosen as an example of a tissue high in transaminase, and liver was used as an example of a tissue whose powers of regeneration would be expected to alter the response of the enzyme.

All data are expressed in terms of the Q_T^{10} unit defined by Ames and Elvehjem (8), μ l. of CO_2 (or pyruvate) liberated/mg. dry wt. of tissue/hr. based upon a 10-min. incubation period.

The data are plotted in Fig. 1. First it may be noted that in the heart tissue, the transaminase level increases apparently due to the excitement and exposure involved in transporting and treating the animals. In the liver this is much less marked. In the heart, the irradiated animals do not show such a marked increase as the control animals, but it is indeed surprising that they show any increase at all. For the first day after irradiation, the irradiated animals were sick and did not eat or drink normally which is reflected in the dry weights of the tissues observed (average controls, heart 28.8% dry wt.; irradiated, 36% dry wt.). After the first day, the irradiated animals seemed normal, the dry weight of tissues returned to normal (25-28%) and, except for a somewhat gaunt appearance, one could see few effects of irradiation. By about the third day after irradiation, the animals became ill and several deaths occurred thereafter, as is noted in Fig. 1. There were only slight signs of hemorrhage and it is our impression that hemorrhage was not the cause of death.

It seems evident, however, that there is little decrease in transaminase activity even in animals ill with radiation sickness and that lack of activity of this enzyme was not the basic lesion in this disease. It seems further that the method described is capable of giving reasonably precise and reproducible results in routine use.

SUMMARY

A colorimetric method for transaminase is described. The application of this method to mice subjected to radiation showed that within the limits of measurement there was no significant decrease in transaminase activity from that observed in nonirradiated controls.

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Amperometric Determination of Soluble Mercapto Groups (Glutathione) in Blood and Tissues¹

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INTRODUCTION

A number of methods for the determination of glutathione (GSH) in biological materials have been described (1-6). Most of these depend upon the titration of a tissue filtrate with oxidizing agents, the most commonly employed being iodine. These methods suffer from two main disadvantages:

(a) Lack of specificity, ascorbic acid being especially likely to interfere.

(b) The so-called dilution effect (7), *i.e.*, the dependence of the result on the iodine/glutathione ratio owing to oxidation beyond the disulfide stage.

In extension of previous work on the amperometric titration of biologically occurring mercapto groups with ammoniacal silver nitrate (8), it has been found that this procedure can be utilized to provide a simple and accurate means of determining soluble mercapto groups (largely glutathione) in blood and tissue which eliminates the above sources of error.

EXPERIMENTAL

1. Preparation of Protein-Free Filtrates

Various protein-precipitating agents such as metaphosphoric acid, acid alcohol, *etc.*, were investigated but were found unsuitable in con-

¹ This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, U. S. Public Health Service.

nection with the amperometric method. Sulfosalicylic acid (1), on the other hand, was found to be very satisfactory for the following reasons:

(a) In the concentrations used it is completely soluble in the alcoholic titration mixture.

(b) It does not interfere with the amperometric silver titration since identical results were obtained for glutathione in pure solution with and without sulfosalicylic acid.

(c) The slight turbidity which occasionally occurs in the sulfosalicylic acid filtrates of some organs does not interfere since removal of the turbidity with methanol causes no significant change in the result (Table I). This is in agreement with the results obtained on metaphosphoric acid filtrates with the iodometric method by Fujita and Numata (4).

TABLE I
*Titration of Glutathione in Rat Liver Filtrate Before and After
Removal of Turbidity*

| Filtrate | GSH ^a added | GSH ^a found | GSH ^a calculated |
|-----------------------|------------------------|------------------------|-----------------------------|
| | mg.-% | mg.-% | mg.-% |
| Turbid | 0 | 196 | — |
| Turbid | 300 | 490 | 496 |
| Cleared with methanol | 0 | 199 | — |
| Cleared with methanol | 300 | 500 | 499 |

^a Glutathione, reduced.

(d) The criticism that sulfosalicylic acid brings ferric iron into solution thereby interfering with the iodometric estimation of glutathione (4) is of course not applicable to the proposed procedure.

(e) In agreement with previous workers (1,5) glutathione was found to be stable for several hours in the presence of sulfosalicylic acid.

Blood Filtrates

Blood was collected from 250-g. rats by cardiac puncture. One volume of blood was laked with 8 vol. of 0.01% saponin and promptly precipitated with 1 vol. of 25% sulfosalicylic acid. Complete hemolysis is essential to release the glutathione from the red cells. The observation of previous workers that blood glutathione is confined to the corpuscles, was confirmed by the amperometric procedure. In order to prevent destruction of glutathione, hemolysis must, however, be carried out as rapidly as possible and this is achieved by laking with a surface-active agent. It was established that saponin and caprylic alcohol do not affect the result. The blood precipitate was

TABLE II
Recoveries of Glutathione from Rat Blood

| | GSH ^a added | GSH ^a found | GSH ^a calculated |
|---------|------------------------|------------------------|-----------------------------|
| | <i>mg.-%</i> | <i>mg.-%</i> | <i>mg.-%</i> |
| Blood A | 0 | 38 | — |
| | 14.9 | 53 | 52.9 |
| | 29.8 | 70 | 67.8 |
| | 59.6 | 99 | 97.6 |
| Blood B | 0 | 39.6 | — |
| | 14.9 | 54.5 | 54.5 |
| | 29.8 | 70 | 69.4 |
| | 59.6 | 96 | 99.2 |

^a Glutathione, reduced.

centrifuged briefly and filtered through hardened filter paper. Aliquots were then titrated as described below.

Recovery experiments were carried out by adding varying amounts of 0.001 *M* glutathione solution to the blood before taking (Table II).

TABLE III
Recoveries of Glutathione From Rat Tissues

| Tissue | GSH ^a added | GSH ^a found | GSH ^a calculated |
|------------------------------|------------------------|------------------------|-----------------------------|
| | <i>mg.-%</i> | <i>mg.-%</i> | <i>mg.-%</i> |
| Liver A | 0 | 209 | — |
| Liver A | 154 | 370 | 363 |
| Liver A | 308 | 513 | 517 |
| Liver B | 0 | 189 | — |
| Liver B | 50.7 | 240 | 239.7 |
| Liver B | 101.4 | 274 | 290.4 |
| Liver B | 202.8 | 392 | 391.8 |
| Liver B | 304.2 | 487 | 493.2 |
| Kidney | 0 | 145 | — |
| Kidney | 150 | 291 | 295 |
| Heart | 0 | 68 | — |
| Heart | 75 | 139 | 143 |
| Spleen | 0 | 118 | — |
| Spleen | 120 | 233 | 238 |
| Small intestine (jejunum) | 0 | 79 | — |
| Small intestine | 75 | 154 | 154 |

^a Glutathione, reduced.

Tissue Filtrates

The animals were killed by a blow on the head; the tissues were removed and weighed quickly on a torsion balance. Portions of the tissue were then ground at 0°C. in an all-glass Ten Brock tissue grinder with 10 parts of 2.5% sulfosalicylic acid. After centrifugation and filtration, as described for blood, most tissues gave completely clear filtrates.

Recovery experiments were carried out by grinding tissue aliquot with 2.5% sulfosalicylic acid containing varying amounts of glutathione (Table III).

2. Titration of the Filtrates

The apparatus and procedure were essentially the same as those described previously (8,9).

Portions of the protein-free filtrates containing from 0.5 to 1.5×10^{-6} equiv. of glutathione were titrated in not less than 80% ethanol with the addition of sufficient ammonium hydroxide and ammonium nitrate to make the concentrations 0.25 *M* and 0.05 *M*, respectively. In addition the titration mixture was made 3×10^{-5} *M* in ethylenediamine tetraacetate. This powerful chelating agent was added to prevent the metal-catalyzed oxidation of glutathione in the alkaline titration mixture.² The mixture was titrated at the rotating platinum electrode with 0.001 *M* silver nitrate solution as described previously (8).

Specificity Studies

A series of amino acids had been tested previously and found not to react with ammoniacal silver nitrate under the conditions used (8). During the present work this was extended to include the rest of all the commonly-occurring amino acids, *i.e.*, leucine, isoleucine, norleucine, valine, lysine, ornithine, aspartic acid, phenylalanine, glycine, alanine, threonine, and cystine. These were tested and found not to react in concentrations ten times greater than those used for the titration of glutathione. In addition, the following compounds were also tested and found not to interfere: adenine, hypoxanthine, xanthine, guanine, uracil, thymine, cytosine, creatinine, uric acid, and coenzyme I.

As pointed out above, ascorbic acid reacts with most of the reagents used for the determination of glutathione, but it was found not to interfere with the amperometric method even when present in a sixfold molecular excess over glutathione, which is considerably greater than the ratio generally encountered in tissues.

² Stock solutions of glutathione can also be stabilized for long periods of time in neutral solution by the addition of this reagent (10).

It should, however, be emphasized that in common with most other methods the argentamine procedure does not distinguish between glutathione and other soluble mercapto compounds, notably ergothioneine, which might be present in blood and tissue filtrates.

RESULTS

Some of the results are shown in Tables II and III. It is evident that good recoveries of added glutathione are obtained with all the tissues used. The accuracy of the method is 1-2%. The values observed for tissues are generally somewhat lower than those found by iodometric and similar procedures (4) which might be accounted for by the greater specificity of the argentamine method. However, the values for blood obtained by this method are within the range of those previously reported (1,2,4,5).

SUMMARY

A simple and accurate method for the determination of soluble mercapto groups in blood and tissues is described. It is shown by means of recoveries that the accuracy of the method is satisfactory. The specificity of the method is of a high order since neither ascorbic acid nor any of the commonly-occurring purines or amino acids (except thioamino acids) react with the reagent under the conditions used.

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Potential Changes in Suspensions of Chloroplasts on Illumination¹

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INTRODUCTION

The investigation of the intermediate steps involved in the photosynthetic process has been difficult because any treatment which ruptures the plant cell membrane results in an almost complete loss of photosynthetic activity. This behavior makes it impossible to use cell-free preparations for experimental work on the over-all process, a technique which has proved of great value for the study of many other metabolic processes. Isolated chloroplasts have been shown to produce extremely small quantities of oxygen on illumination (1), but carbon dioxide is not fixed by such preparations (2).

However, it appears that part of the over-all reaction of photosynthesis can be obtained with isolated chloroplasts in the same order of magnitude as it occurs in living cells. This was first clearly shown by Hill (3,4) who found that the isolated chloroplasts of several species of plants produced oxygen upon illumination if suspended in water extracts of acetone-dehydrated leaves, or in solutions of such oxidants as ferric oxalate. This process is now generally termed the Hill reaction, and proceeds according to the following equation if ferric ion is the oxidant:



Useful oxidants for this reaction include *p*-benzoquinone (5), substituted quinones (6), ferricyanide, chromate, and certain oxidation-

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² Merck Fellow in the Natural Sciences.

reduction (redox) indicators (7). Both intact chloroplasts and chloroplast fragments have been used in the study of the Hill reaction (8). The review of Holt and French (9) includes most of the recent work on this subject.

Previously the rate of the Hill reaction had been measured by observing oxygen evolution (10), the reduction of methemoglobin (11), hydrogen-ion formation (12), and the decolorization of certain redox indicators (7,13). From a consideration of Eq. [1] it would appear that a change in the oxidation-reduction potential of the system should occur as the reaction proceeds. Recently (14) these potential changes have been measured by introducing suitable electrodes into the system and determining the potentials potentiometrically. The present paper presents results of the first general studies of the Hill reaction using this technique.

METHODS

Most of the experiments reported here were carried out with whole isolated chloroplasts of spinach leaves, *Spinacea oleracea*, obtained on the local market. In addition, some work was done with whole cytoplasm preparations of spinach leaves and of leaves of the wild sunflower, *Helianthus annuus* L.

Preparation of Whole Cytoplasm

As a standard procedure 50 g. of washed leaves was blended in 100 ml. of 0.5 *M* sucrose in a Waring Blendor for 30 sec. The resulting semiliquid mass was filtered through several layers of cheesecloth, and the filtrate was centrifuged at low speed for a few minutes. The supernatant contained both whole chloroplasts and fragments of chloroplasts, but practically no intact cells. All of the above operations were carried out at approximately 4°C. in dim light.

Preparation of Isolated Chloroplasts

As a standard procedure 50 g. of washed leaves was blended for 30 sec. in 100 ml. of 0.5 *M* sucrose in a Waring Blendor. The blend was filtered through several layers of cheesecloth and the filtrate was centrifuged for 5 min. at $1000 \times g$. The supernatant was discarded and the sedimented chloroplasts were resuspended in 10 ml. of 0.5 *M* sucrose. Centrifugation and resuspension were repeated several times in order to wash the chloroplasts. (The activity of the chloroplasts was markedly affected by the degree of washing as will be shown later.) The final resuspension was made in a small volume of 0.5 *M* sucrose, and a 0.5-ml. aliquot of this was thoroughly mixed with 9.5 ml. of 75% acetone and allowed to stand in the dark for 15 min. This was then centrifuged for 5 min. at $1500 \times g$ to remove precipitated proteins, and the chlorophyll concentration of the supernatant was determined using a Coleman Junior Spectrophotometer. The chloroplast suspension was then diluted with 0.5 *M* sucrose so that the

final concentration of chloroplast chlorophyll in the irradiation cell was 200–250 mg./l. All of the above described operations with active material were carried out at approximately 4°C. in dim light.

Irradiation Cell

In order to insure reproducible geometry in the illumination process, a plastic cell was constructed with an irradiation chamber 0.25 cm. in thickness and 1.0 cm. in depth, as shown in Fig. 1. The front opening of the cell was covered with transparent plastic and the back opening with platinum foil which also served as one electrode. The cell was assembled with small knurled screws for convenient manipulation. These were threaded into a brass backplate which served to hold the platinum electrode in

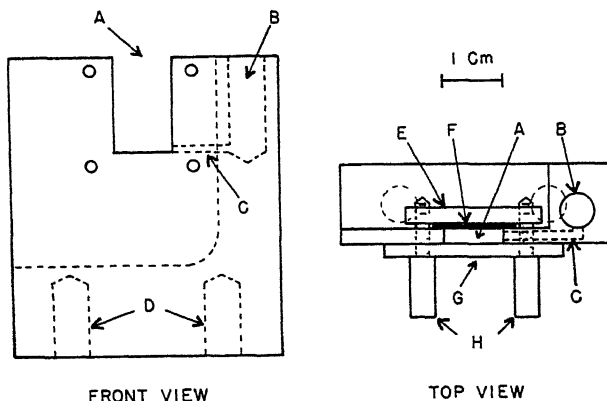


FIG. 1. Diagram of cell for illumination of chloroplast systems. The front view shows the basic plastic block, while the side view shows the rest of the parts in position. A, irradiation chamber; B, side well for calomel electrode; C, hole connecting irradiation chamber with side well; D, holes for mounting cell on locating pins; E, brass back plate; F, platinum foil electrode; G, plastic front window of irradiation chamber; H, brass studs for holding assembly together.

place, and also to make electrical contact with it for convenience in connection. A small hole connected the irradiation chamber with a side well into which a Beckman No. 270 saturated calomel electrode was inserted. The dimensions were such that the addition of 0.5 ml. of material to the cell resulted in a volume of 0.25 ml. in the irradiation chamber, with an active platinum electrode area of 1 cm.² It was considered desirable to have a large electrode area in order to minimize polarization effects, although it has since been found that the platinum surface was not limiting in the measurements even down to an area of 0.1 cm.² The small size of the cell reduced diffusion effects and permitted a decided economy of materials. Unless otherwise specified, the cell was operated in a light-proof box in a refrigerator at 4°C. for use.

Light Source

Light from a 150-w. projection bulb was passed through a condensing and projecting lens system, a glass heat-absorbing filter, a water cell with a depth of 10 cm. and a Wratten A filter (red) which cut off all wavelengths shorter than 5800 Å. The red filter was necessary as white light of the intensity used caused a rapid change in the potential of both Hill's solution and potassium ferricyanide solution. The intensity of red light at the cell surface was approximately 5000 lux for all experiments unless otherwise noted. Light intensity was measured with a commercial meter of low accuracy. Under these conditions the rate of reaction was independent of light intensity. The temperature of the contents of the irradiation cell did not change during illumination, even for exposures of 30 min.

Measurement of Potentials

The platinum electrode and calomel half-cell described above were connected to a Beckman Model G pH meter for potential determinations. Potential could be estimated to 1 mv., and readings could be easily made at 10-sec. intervals. These determinations were found to be very reproducible during the course of a given experiment, as well as from day to day. Potentials are expressed according to the convention described by Latimer (15) in which a calomel half-cell is negative with respect to a normal hydrogen electrode [E° of a saturated calomel half-cell = $-0.2420 + 0.00076(t - 25)$ volts, where t is the Centigrade temperature]. In this convention, if the potential becomes more negative it represents an increase in the relative concentration of the oxidized form of the oxidation-reduction couple controlling the electrode. This is the reverse of the usual convention employed in biology, in which the oxidation-reduction potential becomes more positive when the relative concentration of the oxidized form increases.

PRELIMINARY EXPERIMENTS

Results With Cell-Free Preparations

Whole cytoplasm preparations of sunflower leaves (without added oxidant) prepared as described above showed a rapid potential change on illumination in a direction which indicated that reduction was occurring. When the light was turned off, there was a more gradual change in the opposite direction which leveled off at a value very close to the original potential of the preparation before illumination. This process could be repeated for as long as 12 hr. at room temperature during which time a gradual decrease in the magnitude of the response occurred (14). Activity was immediately destroyed when the preparation was boiled.

This system is of special interest in that it apparently contains a naturally-occurring oxidant, and because the reaction in response to light appears to be reversible in the dark.

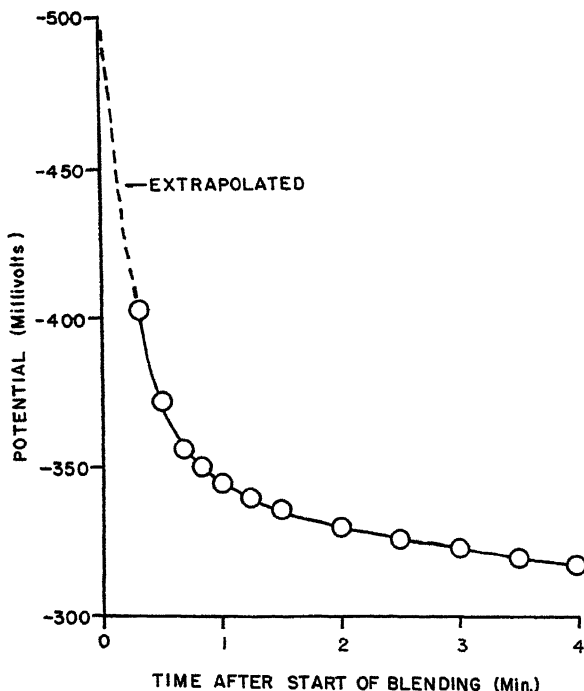


Fig. 2. Curve showing the change in potential with time after start of blending of preparations of spinach leaves.

Whole cytoplasm preparations of spinach leaves prepared as described above show essentially no activity when illuminated. The difference in behavior between the sunflower and spinach whole cytoplasm preparations may be correlated with the observed differences in equilibrium potential values of the two systems. It was found that the potential of whole cytoplasm preparations changed with time. Because of the rapidity of this change in certain cases, leaves were blended for only 5 sec. and then rapidly filtered so that potential determinations could be made within 20 sec. from the start of blending. These determinations were carried out in the dark.

The potential of the sunflower material showed only a small change with time after blending. The potential at zero time (by extrapolation) was approximately -480 mv., and on prolonged standing increased to approximately -440 mv., a change of only 40 mv. The potential of the

spinach preparations on the other hand underwent a large and irreversible change. As is shown in Fig. 2, the zero-time potential (by extrapolation) was approximately -500 mv. which corresponds to that of the sunflower preparations. This changed rapidly with time, however, in a direction which indicated that reduction occurred, and reached an equilibrium after a few minutes at a potential of -250 mv. As the curve shows, the potential changed more than 150 mv. in the first minute after the cells were ruptured, and continued to fall at a decreasing rate. This change apparently occurred in the cytoplasm rather than in the chloroplasts, and was not a result of pH changes because this factor remained essentially constant. If the chloroplasts were separated from whole cytoplasm preparations which had stood for a few minutes, they were found (on resuspension in 0.5 *M* sucrose) to have a potential of -530 mv., while the separated cytoplasm had a potential of -250 mv.

Results With Isolated Spinach Chloroplasts

Isolated chloroplasts of spinach prepared as described above were used for the remainder of the experiments described in this paper. In all of these experiments, the direction of the potential change upon illumination indicated that reduction occurred in the system which dominated the electrode. Suspensions of chloroplasts without added oxidant showed a small potential change on illumination as shown in Curve *B*, Fig. 3. Boiled chloroplasts and colloidal chlorophyll preparations exhibited only very small potential changes upon illumination, even in the presence of added oxidant.

Spinach chloroplasts suspended in Hill's solution (12) (0.02 *M* potassium ferricyanide, 0.01 *M* ferric ammonium sulfate, 0.15 *M* potassium oxalate, and 0.2 *M* sucrose) showed potential changes of as much as 100 mv., as shown in Curve *C* of Fig. 3.

Hill's solution is a rather complicated mixture, and the interaction of the various components upon illumination in the presence of chloroplasts is not precisely defined. Thus it was desirable to use a medium which contained only a single reducible component. Several workers (5,12) have reported that ferricyanide alone functions as a suitable oxidant in place of Hill's solution. This was found to be true in the present work as shown in Curve *D* of Fig. 3, which shows the potential change produced upon illumination of spinach chloroplasts suspended in 0.001 *M* potassium ferricyanide. Quinone was also found to be a suitable oxidant in this system as shown in Curve *E* of Fig. 3.

From a consideration of Eq. [1] it is apparent that relatively large quantities of hydrogen ion will be produced as the reduction of ferricyanide proceeds, and this production of hydrogen ion has even been used to measure the rate of the Hill reaction (12). In unbuffered preparations of chloroplasts, as in the case of all the above experiments, this resulted in a rapid decrease in pH. Since the activity of spinach chloroplasts in the Hill reaction falls off with a decrease in pH, as will be described later, it is apparent that the activity of unbuffered preparations would rapidly decrease with time upon illumination. This probably accounts for the flattening of the voltage *vs.* time curves as shown

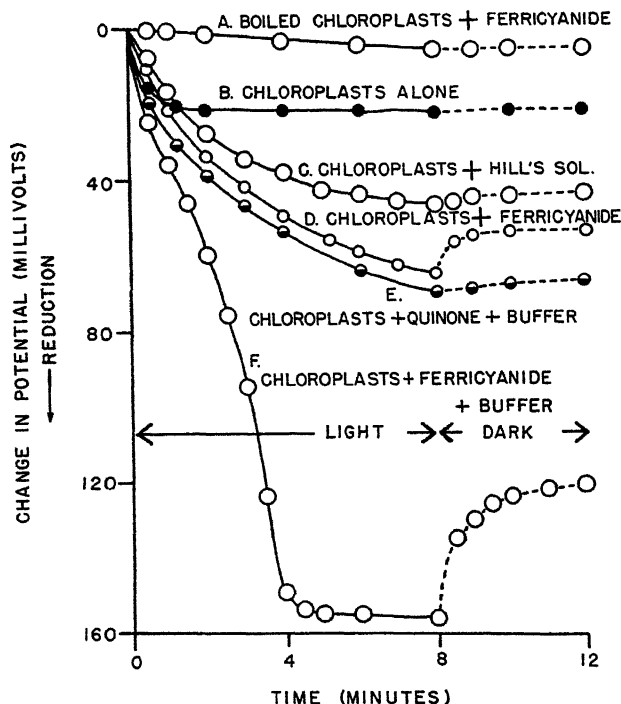


Fig. 3. Curves showing the changes in potential upon illumination of suspensions of washed spinach chloroplasts under different conditions as indicated. Chloroplast concentration equal to 200 mg. chlorophyll/l. Hill's solution as described in text. Potassium ferricyanide concentration 0.001 *M* where used. Quinone concentration 0.01 *M* where used. Potassium phosphate buffer concentration 0.10 *M*, pH 6.85, where used. All data given represent final concentration.

in Curves *C*, *D*, and *E* of Fig. 3. As might be expected, the rapid rate of change of potential on illumination of buffered systems continued for a greater length of time. This is shown in Curve *F* of Fig. 3, where the system consisted of chloroplasts suspended in 0.001 *M* potassium ferricyanide buffered at pH 6.85 in 0.1 *M* phosphate buffer. The shape of the curve also changed from a typical logarithmic decay curve to that of a typical potentiometric titration curve. As a result, all of the systems used in this work were run at pH 6.85 in 0.1 *M* potassium phosphate buffer unless otherwise specified.

Effects of Aging Chloroplasts

Samples of chloroplasts showed a loss of activity with time. Washed chloroplasts stored in the dark at 4°C. lost activity, as shown in Table I.

TABLE I
*Effect of Storing Washed Spinach Chloroplasts Suspended in
0.5 M Sucrose in the Dark at 4°C. on
Their Activity in the Hill Reaction*

| Length of storage hr. | Relative activity |
|--------------------------|-------------------|
| 0.5 | 100 |
| 3.8 | 100 |
| 6.0 | 98 |
| 21.0 | 41 |
| 33.0 | 15 |
| 52.0 | 3 |

The loss in activity with time here is of the same order of magnitude as found by other workers. It was usually possible to use samples from a suspension of chloroplasts for a period of 6 hr. without the necessity of making corrections for loss of activity. As a result, a whole series of determinations could be made with the same chloroplast preparation.

Effect of Washing on Chloroplast Activity

The activity of chloroplast preparations increased if the chloroplasts were washed as described in *Methods*. The effect of successive washings on the activity of chloroplast suspensions is shown in Table II. Further, the activity of washed chloroplasts was markedly reduced by the addition of the cytoplasmic protein suspension. As a result of this behavior,

TABLE II

Effect of Washing with 0.5 M Sucrose on the Activity of Spinach Chloroplasts in the Hill Reaction

| Number of times washed | Activity ^a |
|------------------------|-----------------------|
| 0 | 0.16 |
| 1 | 0.48 |
| 2 | 0.75 |
| 3 | 0.87 |
| 4 | 0.95 |
| 5 | 0.99 |

^a Moles ferricyanide reduced/mole chlorophyll/min.

all chloroplasts used, unless otherwise specified, were washed at least four times.

INTERPRETATION OF POTENTIAL-TIME CURVES

Potential-time curves can be interpreted in terms of the rate of production of ferrocyanide in the following simple manner:

For the reaction: ferrocyanide \rightleftharpoons ferricyanide + e,

$$E = E_c^\circ - \frac{RT}{F} \ln \frac{(\text{ferricyanide})}{(\text{ferrocyanide})}, \quad [2]$$

where E is the observed absolute potential, E_c° is the standard half-cell potential for this redox couple uncorrected for the calomel half-cell reference potential, F is the Faraday of electricity, R is the gas constant, and T is the absolute temperature. If $a = (\text{ferricyanide}) + (\text{ferrocyanide})$,

$$E = E_c^\circ - \frac{RT}{F} \ln \frac{a - (\text{ferrocyanide})}{(\text{ferrocyanide})}, \quad [3]$$

and,

$$\frac{(\text{ferrocyanide})}{a} = \frac{1}{e^{-\frac{F(E - E_c^\circ)}{RT}} + 1}. \quad [4]$$

The standard electrode potential for the ferricyanide system is strongly dependent on potassium ion, less dependent on pH, ionic strength, and other constituents of the solution. Latimer (15) gives the value for this standard potential of the ferricyanide-ferrocyanide system as -0.36 volts but this value must be corrected for the potassium ion of the phosphate buffer and the pH. At 4°C., a pH of 6.8, and a potassium-ion

concentration of $0.10 M$, the measured value was -0.49 volts. At $24^{\circ}C.$, it was -0.44 volts. The dependence of standard potential on potassium-ion concentration is probably due to incomplete dissociation of ferrocyanide (16). In varying buffer and chloroplast mixtures, the standard potential took on a variety of values differing slightly but significantly. Fortunately, each experiment served as a potentiometric titration to provide the appropriate E_s° value. Since E_s° alone appears in the calculations, an absolute determination of E° (standard half-cell potential corrected for calomel half-cell and reduced to unit activity of all reactants and products) is usually not necessary.

In Fig. 4 the potential values of Curve *E*, Fig. 3, are converted to relative ferrocyanide concentrations using the method just described. The figure is a typical result of these experiments and demonstrates a strictly linear rate of ferrocyanide production except at concentration

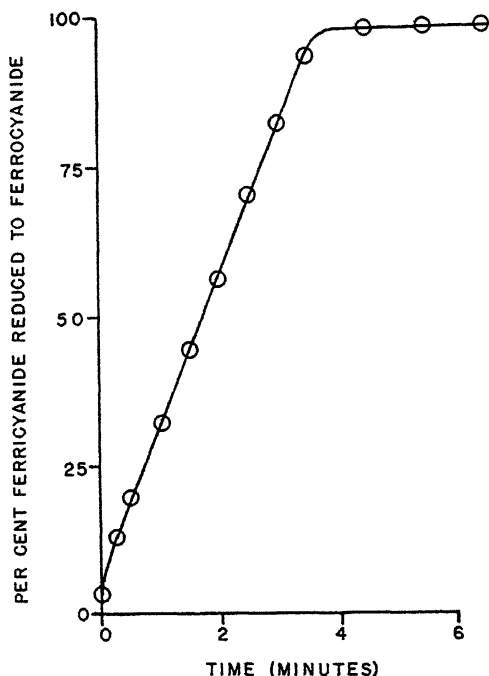


Fig. 4. Curve showing the potential-time data from curve *F*, Fig. 3, plotted according to Eq. [4] in order to show the rate of reduction of ferricyanide with time. The curve levels off sharply at approximately 98% reduction of the ferricyanide.

extremes. The shape of this curve at very low ferrocyanide concentrations is almost certainly the result of ferricyanide reduction by impurities or chloroplast components. At very high ferrocyanide concentrations, the concentration becomes stationary. The latter phenomenon will be discussed in a later section of this paper.

It is apparent from the coincidence of E_r° values in the presence and in the absence of chloroplast and cytoplasm materials that the change in observed potential actually measures the rate of production of ferrocyanide. This conclusion has been verified by direct measurement of ferrocyanide concentrations using colorimetric procedures (17).

There are materials in the cytoplasm able to react with a platinum electrode and able to modify the rate of the over-all reaction in which ferrocyanide is produced. It has been shown that blending of leaf tissue resulted in a rapid increase in reduction potential in the absence of light (Fig. 2). In addition, on first illumination, unwashed chloroplasts showed an increase in reduction potential similar to that observed with whole cytoplasm preparations of sunflowers, but irreversible (Fig. 3, Curve *B*). This increase may well be related to the brief evolution of oxygen observed (1) on illuminating spinach chloroplasts free of artificial oxidant. Washed spinach chloroplasts showed a greater increase in reduction potential on first illumination, and this change was, in part, reversed in the dark. In this case the behavior of the system even more closely resembled that of the sunflower whole cytoplasm preparations (14). It is interesting to note that spinach chloroplasts which had been washed six times still showed this phenomenon even though the concentrations of small molecules able to react at the metal electrode must have been reduced to very low values. Perhaps the small molecules are released from the chloroplasts on illumination, or perhaps the large particles themselves can react in a limited way at the metal surface.

Washing had a marked effect on the activity of spinach chloroplasts as determined by the potentiometric method using ferricyanide as the oxidant. This can be noted from Table II. The activity of a given preparation of chloroplasts was increased several times by repeated washing with 0.5 *M* sucrose. This effect may be due to a natural oxidation-reduction system which serves to buffer the potential of the mixture. The natural system apparently cannot react so readily at the platinum electrode as the ferricyanide-ferrocyanide system, and hence the observed potential is correlated with the latter system. However, in furnishing an additional "sink" for electrons, it would result in lower

apparent rates of ferrocyanide production. In addition, such a natural buffer could control the potential at the electrode in the absence of artificial oxidants. On the other hand, the washing experiments could be interpreted on the basis that an inhibitor for some step in the photo-reduction process was removed.

The constant linearity of rates of reduction of ferricyanide demonstrates that the natural materials in the system did not compete with the added oxidant for electrode control (see Fig. 4). The feature of linear rates also shows that there were no important reactions competing for the ferrocyanide or ferricyanide which were dependent upon the concentration of either of these compounds.

FACTORS AFFECTING THE RATE OF REDUCTION OF FERRICYANIDE

Ferricyanide and Ferrocyanide Concentration

Data illustrating the relation between the initial concentration of ferricyanide in the system and the rate of reduction are presented in Table III. Rate parameters for the reaction as calculated from these

TABLE III
*Effect of Initial Ferricyanide Concentration on the
Rate of Reaction of Ferricyanide*

| Ferricyanide concentration moles/l | Rate ^a |
|---------------------------------------|-------------------|
| 0.0003 | 1.30 |
| 0.00065 | 0.88 |
| 0.0010 | 0.83 |
| 0.0020 | 0.43 |
| 0.0030 | 0.27 |
| 0.0100 | 0.12 |

^a Moles ferricyanide reduced/mole chlorophyll/min.

data show that the rate of reduction of ferricyanide upon illumination of the system follows the rate law:

$$\frac{d(\text{ferricyanide})}{dt} = -4 \times 10^{-1} (\text{ferricyanide})_{\text{initial}}^{-0.8}$$

(the dimensions of the rate constant are moles/min./l.)

These estimates must be considered approximate. The order of the reaction may well be minus first order, though the accuracy of the

experiments fixes the value to only $\pm 30\%$. Reaction rates as calculated from Table III are zero order in ferricyanide concentration as required by the completely linear rates observed for all concentrations. A decreasing reaction rate dependent on the first power of ferricyanide concentration should produce distinct deviations from linearity in individual rate determinations. At present the only explanation which can be offered for the absence of such deviations is that ferricyanide and ferrocyanide are equally effective in inhibiting the action of chloroplast materials.

As predicted by these results, the activity of spinach chloroplasts in Hill's solution, which contains a high concentration of ferricyanide, was lower than in those experiments where ferricyanide alone was used in low concentration. However, the general levels of activity found (about 1 mole of ferricyanide reduced/mole of chlorophyll/min.) were the same as those reported by other workers (8,12). There were some variations in activity of different samples of chloroplasts on a chlorophyll concentration basis, as has been reported (18).

Replacement of ferricyanide by *p*-benzoquinone resulted in a reaction with somewhat different kinetics. The reaction rates were zero order in quinone concentration, but the dependence of such zero-order rates on initial quinone concentration was nearly first order, instead of inverse first order. The anomaly persisted that both reduced and oxidized forms of the added oxidant were equally effective in governing the activity of the chloroplast materials.

Hydrogen-Ion Concentration

The photoreduction of ferricyanide catalyzed by washed spinach chloroplasts was strongly dependent on hydrogen-ion concentration as shown in Fig. 5. The pH of optimum activity and the rate of decrease in activity at lower hydrogen-ion concentrations were in excellent agreement with the observations of other workers (8) using unwashed spinach-beet chloroplast fragments with quinone as oxidant. However, these investigators found a broader maximum and less sharp drop-off in rate as hydrogen-ion concentration was increased. These differences may be due in part to the different materials used. However, our experiments using unwashed or once-washed spinach chloroplasts gave essentially the same curve as that reported by these investigators. This indicates that some cell constituent (probably extrachloroplastic) must

influence the interaction of hydrogen ion with essential chloroplast material. The dependence of activity on pH for spinach and spinach-beet chloroplast material may consequently be identical. On the other hand, our results do not precisely agree with those of other workers (12) who studied whole spinach chloroplasts using a variety of methods.

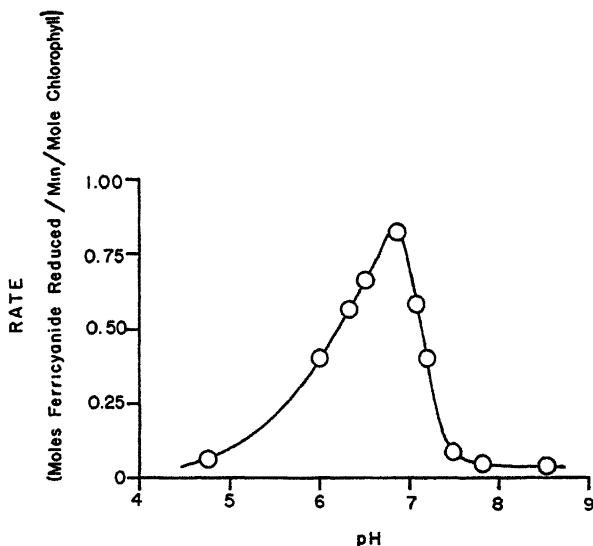


FIG. 5. Curve showing the activity of washed spinach chloroplasts as a function of pH. The maximum activity is at pH 6.85. Potassium phosphate buffer, 0.10 *M*; potassium ferricyanide 0.001 *M*.

No appreciable variations in E_c° are produced by pH changes. At low pH values the chloroplast materials aggregate and settle out of solution leaving an inactive yellowish preparation. There was no immediately observable change in the system at the high pH values where it was inactive.

Effect of Temperature

As expected, considerable inactivation of chloroplast materials occurred at 35°C. In the course of 15-min. experiments at 21°, however, there was no decrease in activity, and the activation energy based on only two points (21° and 4°C.) was 8500 cal./mole ferricyanide. A similar calculation for hydrogen-ion production based on the low-tempera-

ture data of other workers (12) yields a value of 36,000 cal./mole hydrogen ion. The activation energies calculated from the data obtained with chloroplast fragments (8) are 4500 cal./mole ferricyanide and 9000 cal./mole quinone. All of these values must be considered unreliable.

Light Intensity

The photoreduction reaction demonstrated a hyperbolic dependence on light intensity. Light-intensity values were determined by use of a commercial light meter, and are not highly accurate. However, absolute intensities in the region in which the dependence on light intensity changed from zero order to first order were lower than the values reported by other authors [25% in most cases (8,13)]. The agreement among authors is not good perhaps because such large cells were used

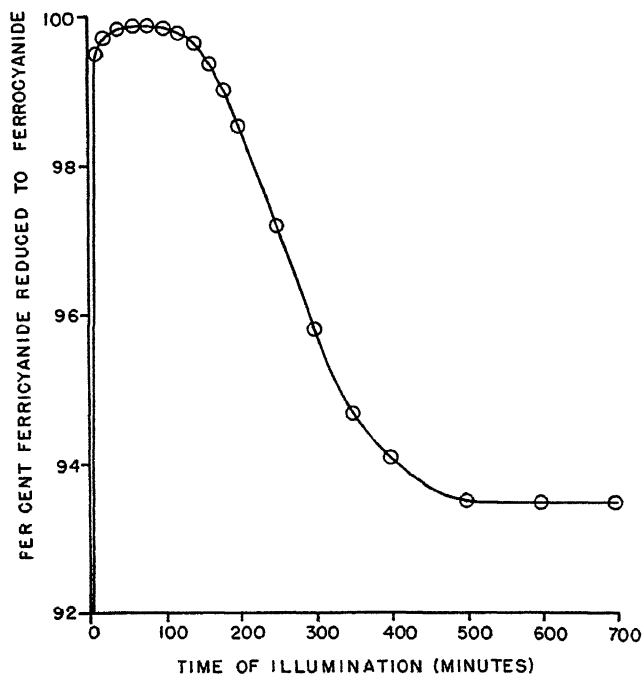


Fig. 6. Curve showing the reduction of ferricyanide by washed spinach chloroplasts during prolonged illumination. Potassium phosphate buffer, 0.10 *M*; pH 6.85. Potassium ferricyanide, 0.001 *M*.

in some cases that not all the chloroplast materials were sufficiently irradiated at low intensities. It is not yet clear whether our results are independent of the size and shape of the reaction cell. It has been reported that light inactivated the chloroplast system (8). The same effect was found in these studies. However, at the light intensities used (5000 lux incident), the inactivation was slow, as is shown in Fig. 6. This curve shows the per cent of ferricyanide originally present reduced to ferrocyanide as a function of time on prolonged illumination. These ferrocyanide concentrations, at least after the first few minutes, are probably the result of steady-state conditions. Hence, the rate at which the ferrocyanide concentration decreases as a function of time probably represents the rate of inactivation of chloroplasts.

Chloroplast Concentration

Since the chloroplast material effective in the photoreduction reaction is almost certainly conserved during reaction, it is to be expected that the reduction of ferricyanide will be of constant order in chloroplast concentration if the geometry of the reaction vessel is such as to allow adequate illumination of all such materials. The data plotted in Fig. 7 show this to be the case over the range of chloroplast concentrations studied. The reaction is seen to be first order in chloroplast concentration as expressed in terms of chlorophyll content. In agreement with observations of other workers (12,18), chloroplast materials prepared on different days from different bunches of spinach showed different activities in the photoreduction reaction. This may well be due to a pretreatment effect on the spinach leaves by illumination, temperatures, etc. As yet, the important factors have not been elucidated.

The Cut-Off Reaction and the Dark Reaction

The rapid fall-off in rate of production of ferrocyanide which distinguishes the ferrocyanide-time curves at low ferricyanide concentrations appears to be closely correlated with the dark reaction, that is, with the decrease in ferrocyanide concentration which takes place when illumination ceases (see Fig. 3). When illumination was stopped, the system remained at very nearly the same potential if the amount of ferricyanide present was very small so that the reaction was nearly stationary; the oxidation potential climbed in the dark to a somewhat higher value than that at which the fall-off in rate took place. On reil-

lumination of the high-ferricyanide systems, reaction took place essentially as though the light had never ceased. Reillumination of the low-ferricyanide systems returned the potential to the cut-off level. The process could be repeated until the material was completely inactivated at which time the potential took on the constant dark value to which it returned in each dark half of the light-dark cycling. These observations suggest that an oxidizing agent is present which can reconvert some

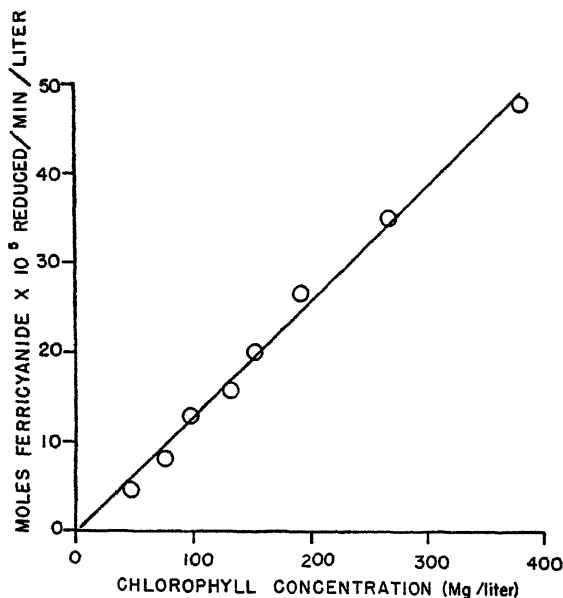
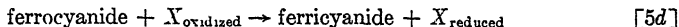
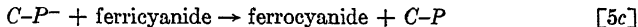
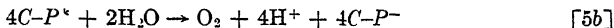
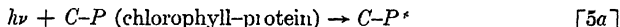


FIG. 7. Curve showing the rate of reduction of ferricyanide upon illumination by washed spinach chloroplasts as a function of chloroplast concentration. Potassium phosphate buffer, 0.10 *M*; pH 6.85. Potassium ferricyanide, 0.001 *M*.

ferrocyanide to ferricyanide independent of illumination. The observations correlating stationary ferrocyanide concentration and chloroplast activity indicate that a dynamic equilibrium exists at high reduction potentials such that the concentration of ferrocyanide remains unchanged so long as the unknown back-oxidation reaction can take place and so long as the chloroplast system is not inactivated. If this unknown back reaction is taken into account, a highly simplified kinetic scheme

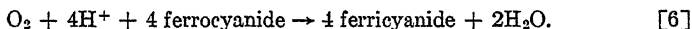
resulting in dynamic equilibrium at high reduction potentials may be written thus:



where X = unknown oxidant in chloroplasts, h = Planck's constant, and ν = frequency of absorbed radiation.

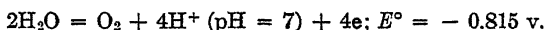
In this series of reactions the simplest possible reaction scheme was selected, and any intermediates which might occur between the specific light-sensitive material of the chloroplast preparations ($C-P$) and the oxidation of water or the reduction of ferricyanide were neglected.

The unknown oxidant must be present in large supply since illuminated chloroplasts in saturated ($\pm 1^\circ\text{C}$.) ferrocyanide solution are not able to change the potential to that of a pure ferrocyanide solution as determined with a platinum electrode. Further, it is seen in Fig. 6 that a large amount of oxidant X must be available either by being present initially or by being produced during the course of ferricyanide reduction. This conclusion follows from the fact that the reducing power of chloroplast suspensions is about 1 mole ferricyanide/min./mole chlorophyll, and the reaction for which the data are plotted in Fig. 6 continued at a high rate for more than 200 min. Under the conditions of the experiment, therefore, 0.05 moles oxidant/l. preparation would be required if one electron is transferred per molecule of unknown oxidant. Pure ferrocyanide solutions react slowly with oxygen, one of the products of the Hill reaction. It seems possible that oxygen is actually the unknown material involved, since it is probably the only oxidant present in sufficient quantity. If such is the case, some substance in the chloroplast suspension must act as a catalyst for the reaction since back oxidation is quite rapid as can be seen by the abrupt change in potential in Curve E of Fig. 3 when illumination is stopped. The back reaction, Eq. [5d], would then be:

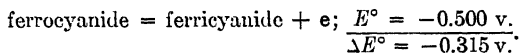


In this case the net result would be a light-sensitive equilibrium, a situation which could explain all present observations.

According to Latimer (15):



We find (at 4°C.):



At a pH of 7 and a temperature of 4°C., neglecting the temperature dependence of the water-oxygen half-cell, the reaction given in Eq. [6] would have an equilibrium constant:

$$K_{O_2} = \frac{(\text{ferricyanide})^4}{(\text{ferrocyanide})^4 (O_2) (H^+)^4} = e^{\frac{4 \times (+0.315)}{0.0239}} = \sim 10^2.$$

This equilibrium constant is of the correct order of magnitude to explain the observed ratio of ferricyanide to ferrocyanide at the cut-off region. Additional experiments in progress will be necessary to establish the precise nature of the cut-off and dark reactions.

DISCUSSION

The experiments presented here are in the nature of a preliminary survey of the uses of the potentiometric method for studying the component parts of the modified Hill reaction. In addition, the experiments with sunflower chloroplast fragments indicate that the technique may be very useful in elucidating some of the characteristics of intermediates in plant cells involved in the transfer of electrons from the photoreceptive system to the subsystem which fixes carbon dioxide. Carbon dioxide fixation has never been shown to occur in cell-free chloroplast cytoplasm preparations. However, marked changes in redox potential have been observed on rupturing the cells and it may be possible, by use of information derived from potential studies, to hold the cell-free system in such a state that the fixation of carbon dioxide could take place. In any event, the rapidity of the technique and its independence on absolute concentration of added oxidant make it a powerful tool in developing the kinetics of the steady- and nonsteady-state mechanisms involved in the photoreduction process and the oxidation of water.

In general, the results presented here verify the findings of other investigators. More detailed experiments are currently in progress.

SUMMARY

1. A new method is described for measuring the reduction of both natural and added oxidants by chloroplasts upon illumination. The

method involves the determination of the ratio of the oxidized and reduced forms of the oxidant potentiometrically using a metallic and a reference electrode.

2. The method was used to investigate the effects of a number of factors on the rate of the reaction, including nature of the oxidant, concentration of the oxidant, age of chloroplasts, washing of chloroplasts, hydrogen-ion concentration, temperature, light intensity, and chloroplast concentration.

3. A highly simplified statement of the basic processes involved in the Hill reaction is presented. Some of the experimental data are interpreted on this basis.

4. The application of the technique to the study of the nature of the oxidants which occur naturally in plant cells is discussed.

5. A rapid change in potential with time on blending whole cytoplasm preparations was observed, and the necessity of controlling this factor in attempts to obtain the over-all photosynthetic reaction in cell-free preparations of plant cells is discussed.

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The Preparation of Ichthyocol Collagen by Electrodeposition¹

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INTRODUCTION

Native collagen may be purified by extensive extractions with salts, organic solvents, and by enzyme treatment (1,2). However, for certain purposes it is desirable to avoid such extensive pretreatment and to obtain a purified collagen quickly and with very mild procedures.

In the course of experiments dealing with ichthyocol collagen it was found that the protein is readily electrodeposited on the cathodal membrane of an electrodialysis apparatus and that this constitutes an easy means of purifying the protein.³ Various methods for the concentration and separation of proteins by electrical means have been described (3). However, none of these involve the electrodeposition on the cathodal or anodal membrane. Since the method is not only convenient in the preparation of collagen but may prove useful also in the case of other proteins the main features of the method are described in this paper.

METHODS AND MATERIALS

The electrodialysis apparatus used in these experiments has been previously described by the author (4). It is an all-glass modification of that described by Albanese (5). It

¹ This paper represents a partial report on research sponsored by the Office of the Quartermaster General, Research and Development Branch, under Project No. 130-46 on "Determination of the Nature and Properties of Skin Structure," under direction of the Leather Subcommittee of the National Research Council Committee on Quartermaster Problems.

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³ The present method is related to the process known as electrodecantation, wherein a colloidal sol concentrates near one end and the bottom of the middle compartment (in the *absence* of stirring) and the supernatant is replaced by decantation.

differs from the vertical type described by Taylor and Iddles (6), in which gravity played an important part (in the separation of amylopectin from β -amylose). In the present apparatus the middle chamber containing the protein is vigorously stirred, the collagen being deposited as a tight skin on the cathodal membrane. A cellophane membrane is used between the middle chamber and the cathodal chamber while a gold-beater skin is placed between the middle and anodal chambers.

The washed tunics of fresh carp swim bladders were extracted with cold 0.05% acetic acid. This causes the disintegration of the bladders and the dispersion of the collagen fibers to form a viscous solution. This solution was filtered successively through Whatman No. 41H filter paper, and coarse, medium, and fine sintered-glass filters. The resulting viscous solution containing 0.1–0.2% collagen was placed in the middle chamber of the electrodialysis apparatus. Rat tail collagen was similarly prepared except that the protein concentration was usually below 0.1%.

The midchamber solution was agitated by a mechanical stirrer while the end compartments, containing water, were stirred by bubbling air through them. Cooling was effected by circulating cold water through glass coils in the end compartments. The electrodes were sheet platinum about 1 cm.² in size.

At the beginning of an electrodialysis, the current rises slowly and reaches a maximum value. The current is never allowed to rise above 25 mamp. in order to prevent the heat degradation of the ichthyocol, which occurs readily above 32°C. in acid solution (7). After some minutes the current begins to fall and is practically zero after 30–60 min. (depending on the collagen concentration), coincident with the complete deposition of the ichthyocol.

EXPERIMENTAL RESULTS

During the course of electrodialysis, protein deposited uniformly over the cathodal membrane. During the process the solution in the cathodal chamber became progressively more alkaline. Since back-diffusion across the membrane is known to occur under such conditions, it is probable that the somewhat elevated pH of the midchamber solution near the membrane may contribute to the precipitation of the ichthyocol collagen (precipitation at or near the isoelectric pH). The deposited collagen was transparent in appearance rather than white and opaque as is collagen precipitated by neutralization or salting out of the acid solution. Examination with the light microscope revealed no characteristic structure but after histological preparation and sectioning in planes parallel and perpendicular to the surface of the membrane, a lamellar structure was apparent. It is believed that this is due to the deposition of very fine ichthyocol filaments, perhaps of the order of 100 Å or less in thickness, as judged by electron microscope observations. It seems probable that the filaments are oriented predominately in planes parallel to the surface but have random orientation within these planes. No pre-

ferred orientation was indicated by observation of the deposited film in polarized light, in a direction normal to the surface. However, stretching the film produced double refraction positive with respect to the direction of stretch, indicating alignment of the filaments. The shearing forces produced by the stirrer may play an important role in determining the orientation. The ichthyocol solution showed strong positive streaming birefringence, indicating that the elongate particles readily align themselves in the flow lines. As deposited, the film was fairly dense and compact. However, when the current was turned off the film began to swell. After peeling the film off the cathodal membrane, it swelled fairly rapidly in distilled water, indicating that any bonds which might have formed between the filaments during electrodeposition must be very weak.

The deposited film was readily soluble in acetic acid. When such a solution was again filtered and electrodialed the ichthyocol again deposited on the cathodal membrane. However, the process was not entirely reversible. After repeating the cycle two or three times, the film became less soluble in acetic acid and eventually became insoluble, through highly solvatable in acid. It may be noted that rat tail tendon collagen does not show the progressive insolubilization of the film; this type of collagen can apparently be repeatedly electroplated without alteration of its acid solubility.

Figure 1 shows absorption spectra obtained with a Beckman spectrophotometer. Curves *B* and *C* are for ichthyocol before and after electro dialysis, respectively. Ordinates are optical density divided by mg. N/ml. (Note the different scale for curve *A* as compared with that for curves *B* and *C*.)

Amino acid analyses [see, for example, the data of Bowes and Kenten (8)] reveal that collagen has a very low content of aromatic amino acids; tryptophan is absent and tyrosine is either absent or has a very low concentration. Since the aromatic amino acids absorb in the region from 250–90 $m\mu$, the absorption spectrum provides a criterion of the separation of the starting material into two fractions.

It will be observed that curve *B* is fairly flat in the region from 250 to 280 $m\mu$, from which it may be concluded that the preparation is relatively low in aromatic amino acids and resembles those reported by Loofbourow, Gould, and Sizer (2) for other collagen preparations. After one electrodeposition the curve is slightly lower than before electro dialysis though the difference is too small to indicate significant purification.

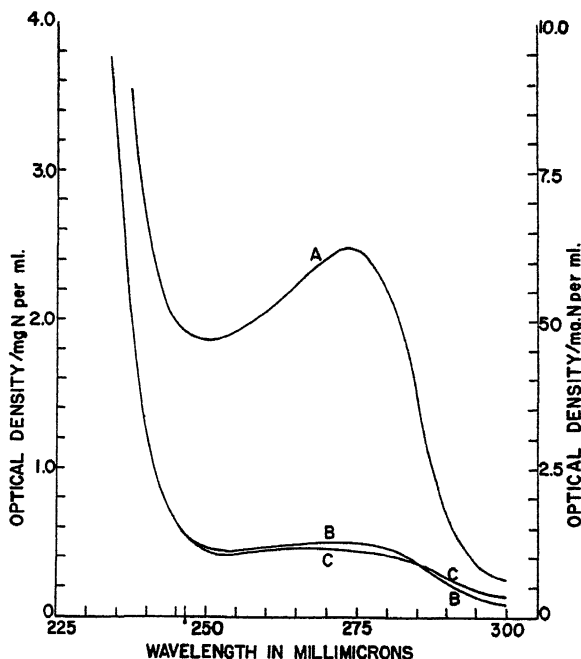


FIG. 1. Ultraviolet absorption spectra of protein solutions. Curve A, concentrated dialysate remaining in midchamber; curve B, original ichthyocol solution; curve C, once plated-out ichthyocol dissolved in aqueous acetic acid. Curve A refers to the right-hand ordinate, while curves B and C refer to the left-hand ordinate.

In contrast with curves B and C, curve A for the midchamber solution (concentrated *in vacuo* over concentrated sulfuric acid at room temperature) after complete deposition of ichthyocol shows high absorption (per mg. N) in the region 250–80 μ . The component responsible for this absorption is soluble at very low ionic strengths and presumably contains aromatic amino acids. No efforts were made to identify the substance more closely. Its possible role in determining the solubility of ichthyocol collagen in acetic acid remains to be determined.

To plate out on the membrane as a coherent gel-like film in electrodialysis, a protein must apparently be fibrous in nature and relatively insoluble at the isoelectric point. Gelatin and albumin solutions do not plate out nor do they precipitate from solution during electrodialysis. A preparation of fibrous insulin⁴ in dilute hydrochloric acid deposited

⁴ Kindly supplied by Dr. David F. Waugh.

on the cathodal membrane only when the solution in the midchamber was unstirred. However, the deposited film had a powdery texture, non-uniform in appearance and lacking in coherence as compared with electrodeposited ichthyocol collagen. Fibrinogen and muscle proteins might meet the conditions for electrodeposition but as yet these proteins have not been investigated.

SUMMARY

Ichthyocol, the collagenous protein of the fish swim bladder, when dissolved in acetic acid can be deposited as a film on the midchamber side of the cathodal membrane of an electrodialysis apparatus. The protein can be redissolved in acetic acid but after several cycles of deposition and re-solution the protein loses its solubility in acid and swells like collagen from certain other tissues.

Ultraviolet absorption spectra indicate that ichthyocol thus prepared is relatively free of material containing aromatic amino acids. A substance probably containing aromatic amino acids remains in the mid-chamber solution after completion of electrodialysis.

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The Partition of Liver Nucleic Acids After Hypophysectomy and Growth Hormone Treatment

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INTRODUCTION

It is suggestive that nucleic acids are concerned with protein synthesis (1-4). Since the anterior hypophysis is known to control body growth (5), it would seem reasonable to assume that the metabolism of nucleic acids must be influenced by hypophysectomy or by injections of the growth hormone. In this paper we wish to report such experiments on nucleic acid partitions in the liver.

EXPERIMENTAL

Male rats (Long-Evans strain) were hypophysectomized at 40 days of age; intraperitoneal injections of the growth hormone were begun on the day of operation and continued for 14 days. A daily dose of 0.2 mg. of the hormone was used. The animals were fasted for 24 hr. before autopsy. Growth hormone was isolated from ox pituitaries by the method previously described (6).

Livers were removed under nembutal anesthesia, exsanguinated and weighed, and placed in a cold chest until the time for the separation of the nucleic acids. Partition was effected by a method modeled after those of Schmidt and Thannhauser (7) and Schneider (8).

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) of the normal controls and some of the hypophysectomized animals were determined by Meijbaum's ribose procedure (9) and by Seibert's modification of the diphenylamine reaction (10), respectively. The results so obtained are given as "mg. of nucleic acids/100 g. of wet liver." Other nucleic acid partitions were obtained from determination of RNA phosphorus (RNA-P) and DNA phosphorus (DNA-P) by the method of Fiske and SubbaRow (11). Such results are expressed as "RNA-P (or DNA-P)/100

¹ Atomic Energy Commission Predoctoral Fellow, 1948-9.

TABLE I
Liver Nucleic Acid Partition in Normal and Hypophysectomized Male Rats

| Experiment | No. of animals | Body weight | | Average liver weight | Nucleic acids/100 g. wet liver | |
|-------------------------------------|----------------|-------------|-----------|----------------------|--------------------------------|-----------------|
| | | Onset | Autopsy | | RNA | DNA |
| Normal rats ^a | 19 | g. — | g. 140 | g. 5.90 | mg. 823 ± 37 ^c | mg. 419 ± 21 |
| Hypophysectomized rats ^b | 8 | 160 | 122 | 3.84 | 702 ± 30 | 466 ± 20 |

^a Male rats 55 days of age.

^b Operated at 40 days of age and 15 days postoperative.

^c Mean ± standard error.

g. extracted liver powder"; the extracted liver powder is that powder from which water, acid-soluble phosphorus, and phospholipide phosphorus have been removed.

RESULTS

As shown in Tables I and II, it may be noted that hypophysectomy causes a decrease in the concentration of liver ribonucleic acids. This difference from the normal value gives a "p" value (13) of 0.05. However, no significant difference was found in the DNA levels. When the

TABLE II
Effect of Growth Hormone Treatment on Liver Nucleic Acid Partition in Hypophysectomized Rats

| Experiment | No. of rats | Body weight | | Average liver weight | P/100 g. extracted liver powder | |
|--|-------------|-------------|-----------|----------------------|---------------------------------|------------|
| | | Onset | Autopsy | | RNA | DNA |
| Normal rats ^a | 4 | g. — | g. 115 | g. 4.60 | mg. 532 | mg. 188 |
| Hypophysectomized rats ^b | 25 | 140 | 117 | 3.99 | 392 ± 24 ^d | 227 ± 25 |
| Hypophysectomized rats plus growth hormone | 33 | 138 | 155 | 4.94 | 450 ± 9 | 205 ± 14 |

^a Male rats 40 days of age; the data were taken from Ref. (12).

^b Operated at 40 days of age and 15 days postoperative.

^c Two-tenths mg. of growth hormone was injected intraperitoneally daily, beginning on the day of operation, for 14 days.

^d Mean ± standard error.

RNA-P concentrations of livers from hypophysectomized animals were compared with those found in the normal animal, a decrease following hypophysectomy is again demonstrable.

The data in Table II indicate that treatment of the hypophysectomized animal with growth hormone results in an elevation of the ribonucleic acid phosphorus concentration toward normal, without any change in the DNA phosphorus concentration. The "*p*" value for this difference is equal to 0.01.

DISCUSSION

The effects of hypophysectomy on the distribution of liver nucleic acids have been previously investigated (14,15). Rapport *et al.* (14) claimed that a small increase in liver RNA was found after hypophysectomy; later they (15) described a fall in the RNA fraction, but no change in the DNA fraction. This is in agreement with the results herein reported. Such a result, along with the increase found after growth hormone treatment, would seem to be in accord with a proposed relationship between nucleic acids and protein synthesis, for hypophysectomy results in a cessation of body growth whereas growth hormone is known to be a protein anabolic agent.

It is possible that the change after hypophysectomy is caused by partial inanition. Davidson and Waymouth (16) have shown that fasting is accompanied by a loss of ribonucleic acids, while the desoxyribonucleic acid content of the liver remains almost constant. A protein-poor diet has also been shown to give the same results (17).

SUMMARY

Hypophysectomy produces a decrease in the ribonucleic acid concentration of rat liver. Growth hormone injection into hypophysectomized animals results in a maintenance of the liver ribonucleic acid concentration near normal.

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On the Mechanism of Enzyme Action. XLIII. Chemistry and Interaction of Lycopersin in the Carbohydrate \rightarrow Fat Conversion by *Fusarium vasinfectum*

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INTRODUCTION

The participation of pigments in the actions of enzymatic systems was demonstrated in 1909 when von Tappeiner showed that the dye hypericin exerted a photodynamic effect on protozoa (1). In the following years several other effects have been attributed to these colored substances, such as influencing the rate of dehydrogenations by molds (2,3), inhibiting the action of certain enzymes (4,5), and possessing antibiotic activity (6,7).

The intervention of the natural *Fusarium* pigments in the carbohydrate \rightarrow fat conversion has been amply demonstrated. When the *Fusarium* pigment, solanione, and naphthoquinones related to it in structure were added to the medium of growing *Fusarium lini* Bolley (F1B), a lowering of the carbohydrate conversion factor (3) and an increase in the desaturation of the fats produced (8,9) were observed. In a radical departure from usual experimental techniques, it has been shown that the presence of the pigment lycopersin (10) in the pigmented mutants of *Fusarium lycopersici* (Flyco) results in the genesis of a less desaturated fat than is found in the unpigmented mutants (11). This change in the desaturation of the fats of unpigmented Flyco was found to be due to an increase in the amount of linolenic acid present.

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³ Communication No. 195. This work was carried out under the auspices of the Office of Naval Research and was aided by a grant of the F. G. Cottrell Fund of the Research Corporation, New York, N. Y.

The pigment isolated from *Fusarium vasinfectum* (Fuvas) shows the same color reactions as lycopersin, as well as an identical absorption spectrum, and therefore it is considered to be identical with lycopersin (12). Since Fuvas could also be grown pigmented and unpigmented, it was of interest again to study the role of lycopersin in the mechanism of the carbohydrate \rightarrow fat conversion in *Fusaria*.

Following the preliminary report on the chemical properties of lycopersin (10), a detailed description of further work will be given at this time.

EXPERIMENTAL

Cultures Employed

The following *Fusaria* were used in these investigations: *Fusarium lycopersici* R-5-6, obtained from the U. S. Dept. of Agriculture, Beltsville, Maryland, through the courtesy of Dr. S. P. Doolittle; *Fusarium vasinfectum* F-273, obtained from the New York Botanical Gardens, through the courtesy of Dr. Wm. J. Robbins.

Fresh isolates of *Fusarium lycopersici* were prepared from tomato plants of the Rutgers variety which were infected by dipping their roots into a suspension of spores of the fungus (13). The ability to produce wilting of tomato plants was used as the chief basis of classification.

The stock cultures of Flyco were maintained on potato dextrose agar, while those of Fuvas were kept on malt agar.

Analytical Procedures

Growing of the Molds. The procedures for growing the *Fusaria*, collecting the fully grown mats, and preparing them for subsequent investigations are the same as those previously described (14).

Twenty flasks were set up in each series of experiments, and at the end of the required time of incubation, the contents were combined for analysis.

Fat and Sterol Determination, Iodine Numbers. The fat and sterol determinations were performed as previously described (14). It should be pointed out that the total lipide and sterol determinations were carried out on chloroform extracts, whereas iodine numbers and spectrophotometric analyses were run on oils isolated by petroleum ether extraction of the mycelia. The extracts were dried over anhydrous Na_2SO_4 , filtered through a sintered glass funnel, and the solvent removed *in vacuo* under nitrogen. Whenever the various fractions of the fats were collected for more than 1 day, they were stored in the cold under nitrogen.

The iodine numbers were determined according to the Hanus method (15).

The sterol determinations required the removal of interfering pigments from the final solutions. This was accomplished in the following manner: 50 ml. of the total 250-ml. chloroform extract was shaken with 10% KOH. The blue emulsions at the interface were separated from the chloroform layers, and the colorless chloroform layers dried over anhydrous Na_2SO_4 , filtered, and then treated in the customary

manner. A blank containing the unpigmented extracts of the *B*-series showed the presence of 2.25% sterol in the untreated sample and 2.37% after treatment.

Spectrophotometric Analysis of the Oils. The oils were isomerized according to the previously used procedure (16) and the earlier described modification for removal of sterols (11) was employed.

Total Pigment Determination. This was carried out by the method previously reported for lycopersin (11).

Quantitative Saponification of Lycopersin. To a weighed sample of lycopersin, 25 ml. of aqueous 0.1 *N* KOH was added by buret. Its titer relative to the standardized H_2SO_4 had been previously determined. The mixture was refluxed under a CaO tube for 5 hr. The cooled solution was titrated with standardized H_2SO_4 to pH 7.0 (glass electrode). After filtration by gravity, the filtrate was made alkaline and distilled. Methanol was identified in the distillate by the chromotropic acid test of Feigl (17).

Analysis: Sample wt.: 0.2948 g., mequiv. KOH 3.11, mequiv./mole (383) 4.02;
 0.1661 g., 1.69, 3.88.

The combined residues were dried in a vacuum desiccator over H_2SO_4 . They were then added to 2 ml. anhydrous pyridine, which was heated to diminish the size of the particles. To the cooled suspension was added 1 ml. benzoyl chloride. After standing for 3 hr. at room temperature, the brownish yellow suspension was poured into ice water. The brown oil was separated from the milky water layer by decantation. On rubbing the oil with ether, it changed to a yellow crystalline material. This was recrystallized twice from benzene.

Analysis: Calcd. for $C_{43}H_{21}O_{10}$: C, 68.63; H, 3.64; OCH_3 , 5.37. Found: C, 68.65; H, 3.76; OCH_3 , 5.97.

Diacetate

To 300 mg. of lycopersin in acetic anhydride was added 2 drops of conc. H_2SO_4 . After a few minutes, the pigment dissolved yielding a dark red solution. This was left in an incubator at 37° for 1 hr. When poured into 50 ml. of ice water, a light-orange oil formed. After rubbing, this changed to crystals. The water was decanted, and the residue recrystallized from benzene to yield a light yellow product, m. p. 281–2°C. (decomp.).

Analysis: Calcd. for $C_{24}H_{19}O_{10}$: C, 61.67; H, 4.07; $2OCH_3$, 13.3; $2COCH_3$, 18.4. Found: C, 61.62; H, 4.31; $2OCH_3$, 13.2; $2COCH_3$, 18.7.

Dibenzoate

To 300 mg. of lycopersin suspended in 5 ml. anhydrous pyridine, 2 ml. of benzoyl chloride was added and the mixture allowed to stand at room temperature. A solid yellow mass formed in a few minutes [contrary to aurofusarin which forms a dark solution (18)]. After 0.5 hr., an additional portion of pyridine was added, and kept at room temperature for 3 hr. more. At the end of this time, the reaction mixture was worked up as described for the saponification product. Bright yellow needles were obtained, m. p. 310°C. (decomp.).

Analysis: Calcd. for $C_{24}H_{22}O_{10}$: C, 69.04; H, 3.89; $2OCH_3$, 10.5. Found: C, 69.32; H, 3.72; $2OCH_3$, 11.2.

Di-p-Nitrobenzoate

To 300 mg. of lycopersin suspended in 5 ml. of dry pyridine was added 500 mg. of *p*-nitrobenzoyl chloride. The reaction mixture was treated as described for the dibenzoate. The yellow product melted at 280°C. (decomp.).

Analysis: Calcd. for $C_{24}H_{21}N_2O_{14}$: C, 59.91; H, 3.08; N, 4.11. Found: C, 59.85; H, 3.39; N, 4.48.

Oxidative Degradation

Two grams of lycopersin in a Soxhlet cup was suspended over 1 l. of methanol-free acetone in a three-necked flask. A reflux condenser was fitted to the neck above the Soxhlet cup, and as the acetone was refluxed it extracted the pigment. To the refluxing mixture, 9 g. of $KMnO_4$ in 300 ml. methanol-free acetone was added dropwise. After 10 hr. the heating was stopped, and an excess of oxidant added. The following day the mixture was decolorized by the addition of methanol, and the MnO_2 filtered off. The acetone was removed *in vacuo* from the filtrate. The residue was suspended in a small amount of water, acidified with conc. HCl, and extracted in a liquid-liquid extractor with ether for 24 hr. After drying over anhydrous Na_2SO_4 the ether was removed *in vacuo*. This left a white substance which was sublimed *in vacuo* at 65°C. After repeating the sublimation, a white product was obtained which melted at 119.5–120.5°C.

Analysis: Calcd. for $C_7H_6O_2$: C, 68.85; H, 4.92. Found: C, 68.4; H, 5.08.

The melting points of the mixtures of the free acid with pure benzoic acid and their corresponding *p*-bromophenacyl esters showed no depression.

Data on Ultraviolet Spectra

All the absorption spectra reported were determined in chloroform solution using a Beckman quartz photoelectric spectrophotometer. The calibration of the wavelength scale was checked by the method outlined by the Bureau of Standards (19).

Data on Infrared Spectra

A Perkin-Elmer model 12a infrared spectrophotometer with a sodium chloride prism was used;⁴ the thermoelectric current was recorded on a strip-chart recorder in the manner previously described (20). The samples were analyzed as Nujol mulls.

Data on X-ray Diffraction Diagrams

The x-ray powder diagrams were obtained with a North American Philips x-ray diffraction powder camera with 57.33 mm. diameter copper radiation filtered through a nickel filter. The pigment was packed in a glass capillary. The diagrams were recorded

⁴ The infrared curves were obtained through the courtesy of Dr. Konrad Dobriner of the Sloan Kettering Institute for Cancer Research, New York, N. Y. The interpretations of the curves were made by Dr. R. Norman Jones of the Division of Chemistry, the National Research Council, Ottawa, Canada.

on 35-mm. Kodak No-Screen X-ray Safety Film, which was developed according to the manufacturer's instructions.⁵

RESULTS

Effect of Lycopersin on Fat Formation in Fusarium vasinfectum

Utilizing the pigmented and the unpigmented saltants of Flyco, it has been shown that the formation of the pigment lycopersin is associated with the genesis of a more saturated fat by this mold, the changes in fat content being due chiefly to the increase in the content of linolenic acid in the isolated oils (11).

A somewhat different situation exists in the case of Fuvas. This fungus normally grows, under laboratory conditions, with very slight pigmentation, the color being limited to the periphery and upper surface of the pellicle. The preliminary experiments designed to demonstrate the best carbon sources for pigment production indicated that very little change in the appearance of the mold occurred with normal carbohydrates, although small differences in the mycelial weights and quantities of pigment were observed. The carbohydrate giving rise to the least amount of pigmentation was lactose. The presence of both galactose and glucose in the culture medium resulted in mycelia of stronger pigmentation, and therefore the disaccharide was used in the following experiments as the carbon source which would induce the formation of an almost colorless mycelium by this mold.

In striking contrast to all the other carbon sources tested for their effects on pigment production, *meso*-inositol⁶ under the identical conditions caused the formation of either a completely pigmented mycelium if the initial pH was acid, or a totally colorless one when the initial pH was adjusted to neutral or alkaline. Thus the possible role of lycopersin in the mechanism of the carbohydrate \rightarrow fat conversion could be demonstrated in another *Fusarium*.

The experimental conditions were so devised that scaled amounts of pigment would be produced depending on the concentration of both lactose and *meso*-inositol, and on the initial pH of the medium. By this method we attempted to attain a gradation in pigment content from

⁵ We appreciate the courtesy of Drs. I. Fankuchen and H. Post, Polytechnic Institute of Brooklyn, New York, in supplying us with the x-ray diffraction patterns presented here.

⁶ We wish to thank the Corn Products Refining Co., New York, N. Y. for providing us with a generous supply of *meso*-inostol.

fully pigmented to lesser amounts, and to approach finally a completely unpigmented form on alkaline *meso*-inositol medium.

In Table I are recorded the variations in the carbon sources and the pH's used to obtain the desired changes. The iodine numbers were determined and the spectral analyses carried out on oils isolated by petroleum ether extraction of the mycelia. The other values were obtained

TABLE I
Changes in the Composition of the Fat of Fusarium vasinfectum

| Series | Carbon source | pH | Mycelial weights | Total lipide | Sterols* | Total iodine value | Pigment in mycelium |
|--------|--|-----|------------------|--------------|-----------|--------------------|---------------------|
| A | % 2.5 Inositol | 3.3 | g. 6.08 | % 19.8 | % 1.17 | 104 | mg./g. 3.48 |
| B | 2.5 Inositol | 7.0 | 6.61 | 20.6 | 2.37 | 157 | 0 |
| C | 2.5 Lactose | 3.3 | 7.45 | 17.6 | 0.86 | 133 | 1.32 |
| D | 2.5 Lactose | 7.0 | 1.31 | 21.3 | 1.01 | 165 | 0 |
| E | 0.75 Inositol + 1.75 Lactose | 3.3 | 7.02 | 14.0 | 0.93 | 150 | 1.96 |
| F | 1.25 Inositol + 1.25 Lactose | 3.3 | 7.25 | 15.1 | 0.83 | 152 | 2.00 |
| G | 1.75 Inositol + 0.75 Lactose | 3.3 | 6.85 | 14.0 | 0.54 | 147 | 1.88 |

* These values may be contrasted with the sterol contents in *Fusarium lini* B. as reported in Ref. (9).

from acidified portions of the mycelia in accordance with the experience of Fink (21) that a preliminary loosening of the fat is required for its more complete extraction. Under these conditions the pigment is also extracted and its presence must be considered in the values recorded for the total lipides.

The formation of lycopersin by Fuvas (Table I) causes the same effect as was observed in the pigmented and unpigmented variants of Flyco. Again, a less desaturated fat is produced in the presence of this pigment. The increase in desaturation in the absence of lycopersin can be attributed to an increase in the quantity of linolenic acid formed as was true for the other mold. This was discerned by the relative heights of the peaks of the ultraviolet absorption spectra of the isomerized oils at 268 $m\mu$.

These general trends are apparent only when comparing those molds grown on the same carbon sources, but at different pH's. The interaction of lycopersin in the mechanism of fat formation is also apparent in the media containing mixtures of lactose and *meso*-inositol. These fluctuations may be due to the fact that in the case of the mixtures, lactose is preferentially utilized for the fat formation, while *meso*-inositol is utilized in the pigment formation.

Conditions for Growth of Fusarium lycopersici; Isolation of Lycopersin

When grown under laboratory conditions, Flyco normally produces a small amount of a red pigment in its mycelium, which changes color to blue-violet as the pH of the medium turns from acid to alkaline. The pigment of *Fusarium oxysporum* has been called a "natural indicator" because of its similar behavior (22). This color formation by Flyco occurs only on a Raulin-Thom medium, and not on a Czapek-Dox medium (3). In a previous attempt to isolate pigment from this particular species (23), only 7 mg. of an impure labile product was obtained after the extraction of 600 g. of dried mycelia. We have found that the addition of trace elements, vitamins, and organic nitrogen to the medium, as well as varying its initial pH did not further the pigment production. However, heavily pigmented mycelia were obtained when a Raulin-Thom medium was inoculated with spore-mycelium suspensions prepared from fresh isolates of the fungus. A similar experience has been reported for *Fusarium Martii-phaseoli* (24).

For the purpose of obtaining the pigment, Flyco was grown on a large scale in both sterilincubators (25) and 3-l. Fernbach flasks. A

Raulin-Thom medium containing 5% glucose was used. At the end of 3 weeks, the blue mycelia were filtered, and then heated in 10% HCl on a steam bath until their color had returned to red (only a small amount of pigment can be removed from the blue form). They were filtered, washed with water, and air-dried. After grinding, the mycelia were first extracted for 24 hr. with petroleum ether (b. p. 40–60°) in a large Soxhlet apparatus to remove most of the fat present. The solvent was then changed to chloroform and the extraction continued for 1 week. At the end of this time, the deep-red extracts were filtered, the solvent removed on a steam bath, and the residues combined. Large quantities of petroleum ether were used for trituration of the residues which were subsequently recrystallized from pyridine. Other suitable recrystallization solvents were found to be anisole and chloroform (the former after supersaturation by extraction of the crude pigment residues in a small Soxhlet).

Lycopersin was also recrystallized from acetone and glacial acetic acid, but an amorphous powder resulted from these solvents. In all cases, when heated the product begins to darken at 250°C., finally decomposing at 305°C.

The pigment obtained from the following sources had identical ultra-violet absorption spectra: unacidified mycelia of Flyco-2, unacidified mycelia of Flyco R-5-6 (both heavily pigmented and mutated forms), acidified mycelia of Flyco R-5-6, and also the mycelia of Fuvas. In addition, the absorption spectra of the dibenzoates (Fig. 1) prepared from pigments isolated from the blue and the acidified red mycelia were identical. Further proof of the homogeneity of this material were the identical absorption spectra of samples recrystallized from pyridine, anisole, or chloroform (12).

The absorption maxima of lycopersin are located at 266, 272, and 522 $m\mu$ (Fig. 1).

Chromatography of lycopersin on alumina or Florisil resulted in the formation of a violet area at the top of the column. This color change is accompanied by salt formation, and it was found impossible to elute the material from the adsorbents.

Structural Study of Lycopersin

Elementary analysis of lycopersin shows that it does not contain elements other than carbon, hydrogen, and oxygen. The microanalyses of the pigment indicated an empirical formula of $C_{10}H_8O_4$.

Analysis: Calcd. for $C_{10}H_8O_4$: C, 62.50; H, 4.17; OCH_3 , 16.2. Found: C, 62.67; H, 3.81 (average of 6 analyses); OCH_3 , 15.6.

Refluxing the pigment in aqueous KOH produced a deep-violet solution. Methanol was identified in the distillate. This saponified pigment was insoluble in 5% $NaHCO_3$, and formed a methoxyl-containing dibenzoate. Since one methoxyl group was lost as methanol and the other retained, the empirical formula proposed for lycopersin is $(C_{20}H_{15}O_8)_n$.

The color and solubility changes of lycopersin with aqueous alkali solutions indicate the presence of an acidic hydroxyl group. A diacetate, dibenzoate, and di-*p*-nitrobenzoate were prepared, and their analyses agree with the proposed formula.

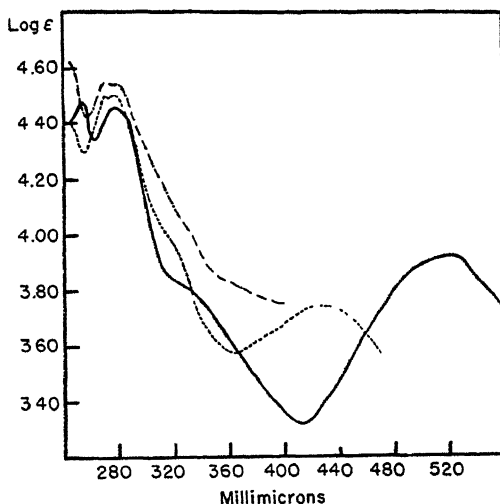


Fig. 1. Ultraviolet absorption spectra (in chloroform) of: — lycopersin, lycopersin diacetate, - - - lycopersin dibenzoate.

The absorption spectrum of the diacetate shows maxima at 270, 276–280, and 426–430 $m\mu$ (Fig. 1). That of the dibenzoate has maxima at 272 and 280 $m\mu$ (Fig. 1).

Although our analytical data agree with the proposed formula it must be remembered that the actual formula may be an integral multiple of that set forth. The Rast method could not be used for the determination of the molecular weight, for the desired concentrations of the pigment in both camphor and borneol could not be achieved.

In addition, the derivatives decompose at the temperatures necessary for this determination. Ebullioscopic methods could not be resorted to because the pigment is not sufficiently soluble in the solvents in which these determinations are usually made. On x-ray diffraction of the largest single crystals available (the platelets obtained by Soxhlet extraction with chloroform), clear patterns could not be picked up despite a 24-hr. exposure.

Lycopersin obtained by pyridine recrystallization consists of needles, while the samples obtained by chloroform Soxhlet extraction are platelets. The polymorphism (26) of these crystals is further evidenced by their x-ray powder patterns (Fig. 2). A similar observation has recently been reported for the semicarbazones of 5-nitro-2-furaldehyde (27).

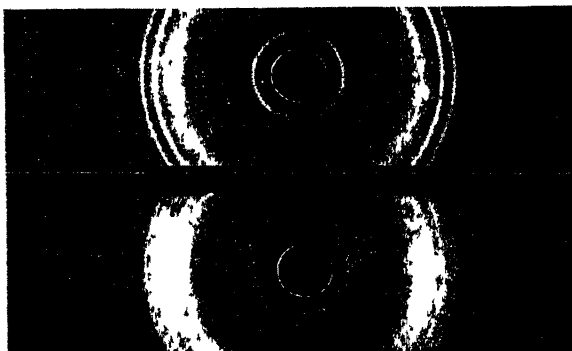


FIG 2. X-ray powder patterns of lycopersin: needles from pyridine (above), platelets from chloroform (below).

This compound crystallized in two forms depending on the solvent used. The analyses and ultraviolet absorption spectra of these semicarbazones were identical, yet their x-ray powder diagrams differed markedly from each other.

Attempts to determine the nature of the four remaining oxygen atoms were unsuccessful. Although there were indications of the formation of an oxime, clear-cut carbonyl derivatives could not be isolated. Despite the color changes of the compound with alkalis, it does not dissolve in them. From this it was concluded that carboxyl groups are absent. To clarify this aspect of the problem, the compound was subjected to infrared absorption analysis. The interpretation of the spectra was the following:

Hydroxyl Region. The infrared absorption spectrum in this region shows no positive indication of the presence of the hydroxyl group in either lycopersin or its acetate. This does not exclude the presence of an associated hydroxyl group, as other cases have been observed in which molecules known to contain hydroxyl groups do not exhibit the characteristic band (28).

A Region ($4000\text{--}2530\text{ cm.}^{-1}$). At 3070 cm.^{-1} (approximately) there is a small band present in lycopersin but absent from the acetate. This indicates the presence of an unsaturated system containing $=\text{C}-\text{H}$.

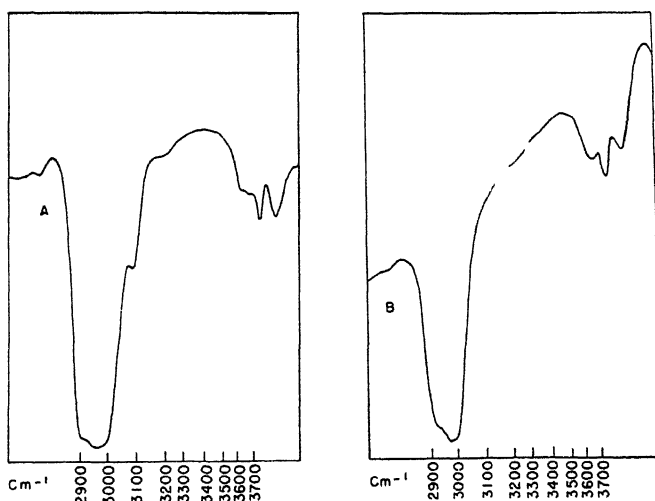


FIG. 3. Infrared absorption spectrum in the "A" region: A, lycopersin; B, lycopersin diacetate.

B Region ($2000\text{--}1620\text{ cm.}^{-1}$). There is a strong band present in the lycopersin acetate at 1769 cm.^{-1} . This is higher than frequently expected for a secondary acetate, but is suggestive of an acetate of a phenolic hydroxyl.

In the diacetate there are other bands present at 1649 cm.^{-1} , 1670 cm.^{-1} , 1627 cm.^{-1} and at 1580 cm.^{-1} . The bands at 1627 cm.^{-1} and at 1580 cm.^{-1} are frequently observed in compounds containing aromatic rings. In lycopersin, the bands at 1769 cm.^{-1} and 1649 cm.^{-1} are absent.

The infrared "fingerprint" regions of the two compounds are presented in Fig. 5.

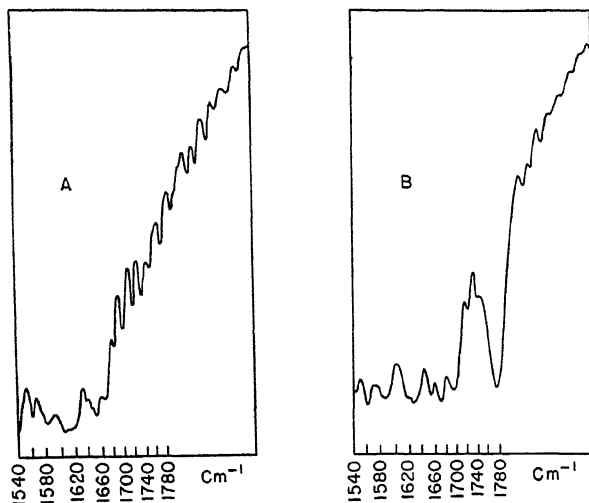


FIG. 4. Infrared absorption spectrum in the "B" region: A, lycopersin; B, lycopersin diacetate.

The infrared absorption spectral indications of the existence of aromaticity are confirmed by the isolation of benzoic acid as an oxidative degradation product of lycopersin.

Attempts to show the presence of a quinoid group in the molecule by reductive acetylation (29) resulted in the formation of the previously obtained diacetate. Reductive benzoylation (30) was also of no

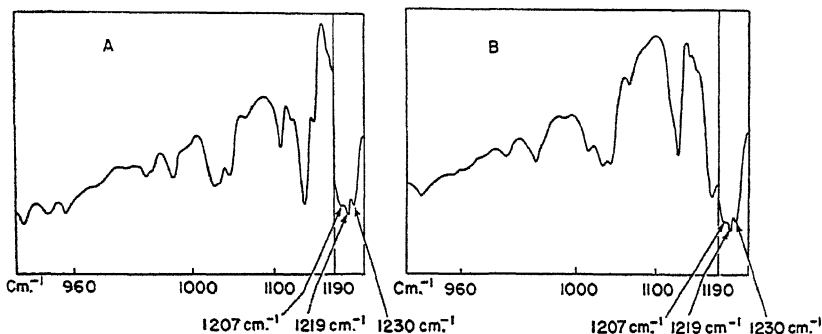


FIG. 5. Infrared absorption spectrum in the "fingerprint" region: A, lycopersin; B, lycopersin diacetate

avail. When treated with LiAlH_4 in dioxane (31), HI, and red P (32), and by the modified Wolff-Kishner method (33), a reduced product could not be isolated. When subjected to hydrogenation using both Raney Ni and Pd-C as catalysts in various solvents at 40 p.s.i. in a Parr apparatus and at 1500 p.s.i. in an Aminco high pressure bomb, at room temperature and at 150°C ., the color of the pigment was neither changed nor discharged.

Antibiotic Activity

A red pigment to which has been assigned the same empirical formula as lycopersin has been isolated from *Penicillium nigricans-janczewskii* (34). The two pigments also have the same methoxyl contents. However, they are undoubtedly different for they display different ultra-violet and infrared absorption spectra (35).

The antibacterial activities of Flyco and Fuvas were determined against gram-positive and gram-negative bacteria (36). On both nutrient agar and asparagine dextrose agar there was no inhibition of the growth of these organisms. However, lycopersin and its diacetate exhibited partial inhibitions of the growth of *Bacillus mycoides* and *Micrococcus pyogenes* var. *aureus*.

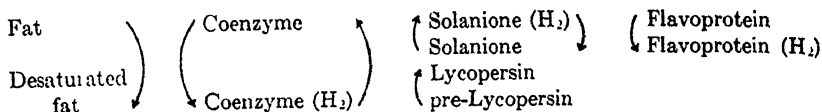
DISCUSSION

The presence in *Fusaria* of dehydrogenases capable of acting on both saturated and unsaturated fatty acids has been demonstrated (37). When solanione, nicotinic acid, or riboflavin were added to the medium of growing FIB, a fat more desaturated than that of the control was formed (11). Since nicotinic acid and riboflavin are reversibly reducible and are known to be integral components of some coenzyme systems operative in hydrogen transport, by the same token, solanione may participate in the hydrogen transport by virtue of its reversible reducibility both *in vitro* and *in vivo* on the basis of its quinoid nucleus and redox potential (38).

In contrast to the action of solanione in growing FIB, the formation of lycopersin by the cells of Flyco and Fuvas is accompanied, though not quantitatively, by genesis of a less desaturated fat than is formed by the unpigmented cells. Since this pigment is not reducible by the usual chemical methods and is more saturated with respect to hydrogenation catalysts, it would seem that the fatty acid dehydrogenases operative in *Fusaria* carry the hydrogen from the hydrogen donor in part to a "precursor" of the pigment. Due to the latter's nonreversible

reducibility, further transfer of hydrogen from the pigment does not take place. This static behavior of lycopersin in the action of the fatty acid dehydrogenase system gives rise to a more saturated fat, while its absence in the unpigmented variants results in a fat of greater desaturation. This is also evidenced by the ratio of the absolute amounts of the pigment present and the total lipides formed in each mycelial mat.

These considerations are illustrated by the following scheme:



Since lycopersin is a terminal product and incapable of acting as a transfer agent, further desaturation of the fat does not occur. The well known broad pH range of some members of the genus *Fusarium* furthermore supports the contention that the recorded observation of the changes in fat composition are attributable to the presence of the pigment rather than to environmental variations.

It has been shown that acetaldehyde can presumably function as the key intermediate (39) in the conversion of glucose to fats, sterols (40), and pigments (41) in *Fusaria*.

Unusual results with *Fuvas* were obtained when *meso*-inositol was used as the carbon source. When the mold was grown for 9 days on 5% lactose (pH 3.4, Raulin-Thom), and then the medium removed, the mycelium washed with sterile distilled water, and fresh Raulin-Thom medium containing 5% *meso*-inositol (pH 3.4) added, heavy formation of pigment occurred only 2 days later. If the carbohydrate was replaced by *meso*-inositol in a Raulin-Thom solution at pH 7.0 then no pigment formation was observed. In yet another case, when the mold was pregrown on *meso*-inositol at pH 7.0 and the medium changed to *meso*-inositol at pH 3.4, then the formation of pigment likewise occurred.

From this experiment it is evident that *meso*-inositol is an extraordinary starting compound for pigment formation. Although the literature abounds in speculations concerning the possible cyclization of hexoses to inositol to form aromatic products in nature (42), practically nothing is known of the mechanism of utilization of this pseudo-carbohydrate (43). The isolation of two oxidation products of *meso*-

inositol by *Acetobacter suboxydans* with the ring still intact has been reported (44,45). This reaction could be considered as analogous to a mild *in vitro* oxidation. It does not furnish any further insight into this problem.

In the utilization of *meso*-inositol by Fuvas, certain observations were made: the ultra-violet spectroscopic absorption of the free oils showed the presence of sterols (46), while the isomerized oils had the typical absorption spectra of the straight chain unsaturated acids (47). *meso*-Inositol was also the best carbon source for the formation of lycopersin. It is evident that in its utilization by Fuvas, not only aromatic nuclei are formed, but also the condensed ring systems of steroids. In addition, a splitting of the ring must occur to allow for the formation of straight-chain fatty acids. Therefore, this compound does not seem to be merely aromaticized, but in its utilization it must be degraded, as other carbohydrates are, to a smaller intermediate to account for the formation of such a wide diversity of products. The reactive intermediate may presumably be acetaldehyde, for this compound has been indicated to function in the formation of ergosterol (40), *p*-methoxy methyl cinnamate (48), and solanione (41) in fungi.

Our present experiments extend the earlier reported observations (8,9) that natural pigments and related compounds depending on their structure are able to interact in the mechanism of the carbohydrate → fat conversion.

The literature records far-fetched attempts to bring biochemical reactions to a grossly simplified common denominator (49). The outdated concept that fungal pigments are unphysiological waste products has also been recently revived (50). The recorded participation of *Fusarium* pigments in enzymatically governed reactions again shows that the above considerations are contrary to facts.

SUMMARY

1. The presence of the pigment lycopersin in *Fusarium vasinfectum* apparently interferes with the dehydrogenation of the saturated fat. The increase in the unsaturation of the oils is attributed to an increase in linolenic acid content.

2. A method for the isolation of the pigment lycopersin is given, and evidence for its being a single entity is presented.

3. On the basis of the microanalytical elemental analyses of the four derivatives of the pigment, it is believed to be $C_{20}H_{18}O_8$, or an integral

multiple of this. It is a dihydroxy, dimethoxy compound containing a phenyl radical.

4. A possible mode of action of lycopersin in the carbohydrate \rightarrow fat conversion in *Fusaria* is discussed.

5. Experiments on the utilization of meso-inositol by *Fusarium vasinsectum* indicate that the molecule is broken down to a reactive intermediate, rather than directly aromatized to form complex products.

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Distribution of Thiamine and Riboflavin in Components of Blood

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INTRODUCTION

In spite of many reports concerning the thiamine and riboflavin content of total blood, little is known about the vitamin content of each blood component, especially leucocytes and platelets, possibly owing to the difficulties of isolating them. Goodhart *et al.* (1) reported the very high content of thiamine in the leucocyte layer of blood from a patient with myeloid leukemia containing 124 $\mu\text{g.}/100\text{ ml.}$, whereas that from a patient with lymphatic leukemia gave a value of 17.5 $\mu\text{g.}/100\text{ ml.}$ Abels *et al.* (2) studied extensively thiamine metabolism in leukemia. Eighteen cases of myeloid leukemia averaged 296 $\mu\text{g.}$ thiamine/100 ml. white blood cells. Fourteen cases of lymphatic leukemia averaged 270 $\mu\text{g.}/100\text{ ml.}$ Thirty normal individuals averaged 99.8 $\mu\text{g.}/100\text{ ml.}$ The thiamine content of platelets was found to be only 10% less than that of leukocytes. Shimazono (3) reported also similar cases with thiamine content of 150 $\mu\text{g.}/100\text{ ml.}$ of total blood of myeloid leukemia. From these facts it is suggested that leukocytes contain a very large quantity of thiamine. Suzawa (4) determined not only the thiamine content of erythrocytes and plasma, but also that of the leucocytes from normal subjects and those with myeloid leukemia. He reported the value of about 30 $\mu\text{g.}/100\text{ ml.}$ for the former, and 100–160 $\mu\text{g.}$ for the latter. But he did not succeed in separating completely the leucocytes from platelets. The complete isolation of platelets is rather easy [cf. Fujita (5)], but, so far as we know, the complete isolation of leucocytes from intermingled erythrocytes has not yet been achieved. After several attempts we succeeded in accomplishing this, and we determined the vitamin content of each component of blood and the distribution of vitamins in whole blood.

ISOLATION OF BLOOD COMPONENTS

Isolation of Plasma and Erythrocytes

One and one-tenth per cent sodium citrate solution in 0.75% NaCl was prepared, and 1 ml. of this solution was added to 3 ml. of blood and mixed gently. Plasma and cells were separated by centrifugation. By inserting a pipet into the cell layer, one can remove desired amounts of erythrocytes, which are stored suspended in plasma. Before determination, the plasma is replaced by saline solution.

Isolation of Platelets and Leucocytes

At the interface of the plasma and erythrocyte layer there appeared a greyish white layer consisting mainly of platelets, and a rose-colored layer of leucocytes inter-

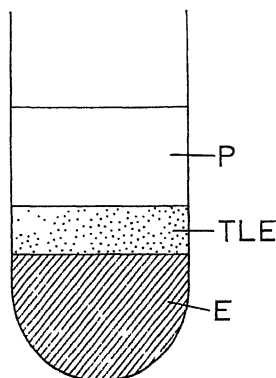


FIG. 1. Separation of blood components. P, plasma; T, platelets; L, leucocytes; E, erythrocytes.

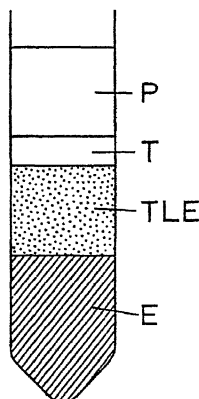


FIG. 2. Collection of white cell layer. (Identification as in Fig. 1.)

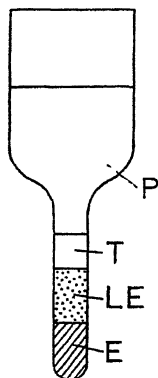


FIG. 3. Separation of leucocyte and platelet layers. (Identification as in Fig. 1.)

mingled with erythrocytes (Fig. 1). These intermediate layers from many tubes were pipetted into a conical centrifuge tube containing plasma and were centrifuged (Fig. 2). The white and the rose layers, after pipetting into a hematocrit tube, were centrifuged (Fig. 3). The white layer consisting mainly of platelets was collected in a tube containing plasma. The rose layer, consisting mainly of erythrocytes and leukocytes, after collection in a hematocrit tube was centrifuged. The remaining white platelet layer and the erythrocyte layer as well as the plasma were pipetted off. Hemolysis of residual red cells was accomplished by the addition of 1% acetic acid. After a brief centrifugation, the hemolyzed solution and stroma were pipetted off (Fig. 4). The leukocytes were suspended in plasma. For determination of thiamine and riboflavin, the plasma was replaced by physiological saline solution.

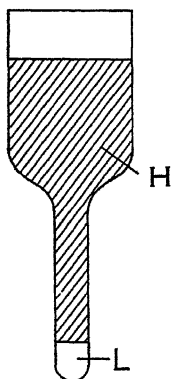


FIG. 4. Hemolysis of leucocyte layer. H, hemolyzed solution; L, leucocytes.

EXPERIMENTAL DATA

The human blood was collected from three healthy male youths with blood type O. The blood of each animal was collected aseptically soon after killing. For one complete determination 200 ml. of blood was necessary.

Determination of Thiamine

Thiamine was determined after enzymatic hydrolysis and permutit treatment by the improved thiochrome method (5). Free thiamine was scarcely demonstrable in any of the blood components. For calculation of the vitamin contents of total blood and plasma, the dilution of 4:3 was taken into consideration. The volumes of cells were determined in hematocrit tubes. To ascertain whether the acetic acid treatment may cause any loss of thiamine in intact leucocytes the following experiments were performed.

TABLE I

Content and Distribution of Thiamine and Riboflavin in Components of Blood
(With standard deviation: $\sigma = \sqrt{\sum f d^2/n}$)

| Species | Vitamin | Cases | Plasma | Erythrocytes | Platelets | Leukocytes | Whole blood |
|---|----------------|-------|----------|--------------|-----------|------------|-------------|
| Vitamin content in $\mu\text{g.}/100$ ml. of component | | | | | | | |
| Horse | B ₁ | 8 | 2.5±0.1 | 9.0±0.5 | 74±4.4 | 59±6.2 | 6.4±0.3 |
| | B ₂ | 2 | 3.6±0.3 | 12.3±0.3 | 163±7 | 156±9.5 | 8.5±0.7 |
| Cattle | B ₁ | 2 | 2.4±0 | 9.6±0.5 | 60±1.3 | 51±2.8 | 6.4±0.2 |
| | B ₂ | 2 | 4.3±0.1 | 23.3±0.1 | 185±13 | 151±4.5 | 16.7±0.4 |
| Swine | B ₁ | 2 | 2.8±0.5 | 17.9±0.1 | 31±1.1 | 29±0.3 | 9.9±0.2 |
| | B ₂ | 2 | 11.6±0.4 | 40.4±2.4 | 205±3 | 172±5 | 27.8±1.1 |
| Goat | B ₁ | 1 | 2.4 | 8.2 | 31 | 28 | 6.0 |
| | B ₂ | 3 | 1.7 | 11.1 | 72 | 47 | 7.0 |
| Man | B ₁ | 3 | 3.1 | 10.6 | 120 | 115 | 6.9 |
| | B ₂ | 3 | 3.1 | 10.6 | 120 | 115 | 6.9 |
| Distribution of vitamin in $\mu\text{g.}/100$ ml. of blood ^a | | | | | | | |
| Horse | B ₁ | 8 | 1.5 | 3.5 | 0.5 | 0.4 | 5.9 |
| | B ₂ | 2 | 2.1 | 4.7 | 1.1 | 1.1 | 9.0 |
| Cattle | B ₁ | 2 | 1.3 | 4.2 | 0.4 | 0.3 | 6.3 |
| | B ₂ | 2 | 2.5 | 9.6 | 1.5 | 1.4 | 15.0 |
| Swine | B ₁ | 2 | 1.7 | 7.1 | 0.4 | 0.4 | 9.5 |
| | B ₂ | 2 | 6.6 | 16.7 | 1.5 | 1.4 | 26.2 |
| Goat | B ₁ | 1 | 1.4 | 3.6 | 0.3 | 0.3 | 5.6 |
| Man | B ₁ | 3 | 1.0 | 4.5 | 0.7 | 0.5 | 6.6 |
| | B ₂ | 3 | 1.9 | 4.0 | 0.7 | 0.8 | 7.4 |

^a Calculated from cell volumes and the vitamin analyses of each component.

The thiamine content of leucocytes, determined after standing for 30 min. in 1% acetic acid after hemolysis, and those determined immediately after hemolysis, were practically the same. Therefore in the main test, in which the leucocytes were treated with 1% acetic acid and the acid was replaced immediately after hemolysis by blood plasma, any loss of thiamine during the process may hardly be questioned.

Determination of Riboflavin

Riboflavin was determined by the improved lumiflavin method [cf. Fujita (6)], the accuracy of which has been proved by numerous estimations. Through benzyl alcohol treatment it was demonstrated that more than 95% of riboflavin in blood exists in esterified form (7).

Distribution of Vitamins in Each Blood Component

From the volume per cent and vitamin content of each blood component the total amount of thiamine and riboflavin in blood was calculated. The calculated values agree fairly well with the values directly determined.

The vitamin content and distribution of each blood component are shown in Table I. In this table only average values are shown. The individual values agreed within 10% of the averages.

DISCUSSION

Westenbrink (8) *et al.* determined thiamine in whole blood of various species manometrically and found following values in $\mu\text{g.}/100\text{ ml.}$ Ox (14): 5.9 ± 1.3 ; calf (5): 9.3 ± 1.3 ; sheep (11): 7.3 ± 1.7 ; pig (10): 19.4 ± 4 ; horse (1): 5.6 ; rat (6): 17.2 ± 6.3 ; man (2): 11.2 ± 1.5 .

The results on the species in common differ considerably from the results above reported. However, this is not surprising because of differences in diets as well as differences in method.

The values for riboflavin content of human blood obtained by the authors are lower than those obtained by a micro procedure of Burch *et al.* (9) who found that white blood cells contain $252\ \mu\text{g.}/100\text{ ml.}$, red blood cells $22.4\ \mu\text{g.}/100\text{ ml.}$, and serum $3.2\ \mu\text{g.}/100\text{ ml.}$ of riboflavin. The differences may be due either to differences in test subjects or probably to differences in method. The method which was used is based on fluorometry of the substance reducible by hydrosulfite. It is possibly not so specific for riboflavin as lumiflavin.

SUMMARY

1. The complete isolation of platelets and leucocytes was achieved. The method of isolation is described in detail.
2. The volume per cent of each blood component, thiamine and ribo-

flavin content in 100 ml. of each component and in 100 ml. of whole blood were determined. The thiamine and riboflavin content of leucocytes and platelets was found to be high. Since about 1% of each of these components is found in blood, their contribution to the total quantity is only 10% to 25%. Fifty per cent to 70% was contained in erythrocytes and the remainder in plasma.

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Biochemical Studies on *Penicillium chrysogenum* Q-176.

I. Phosphatase Activity and the Role of Zinc in the Production of Penicillin

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INTRODUCTION

The production of penicillin is dependent on various factors such as the efficiency of the strain used, the nature and composition of the culture medium, the temperature of incubation, the rate of aeration, and the density of the inoculum. It has now been clearly established (1,2) that even when other conditions are equal, only certain strains can produce a high concentration of penicillin and that such a strain is capable of the highest efficiency as a penicillin producer only when the culture medium supplies all its nutritional requirements in optimum concentrations. This latter aspect has been studied in some detail by Foster, Woodruff, and McDaniel (2) and it has been observed that for proper and efficient production of this antibiotic, fairly high concentrations of phosphates and corn steep liquor are required. In addition, an optimum concentration of some of the trace elements is also necessary. Of these elements, zinc appears to be important; in both high and low concentrations, it has been observed to lessen the efficiency of formation of penicillin by the mold. The indispensability of this element in the nutrition of the mold is explained by the fact that zinc is necessary for the rapid oxidation and assimilation of sugar and to induce the pH to rise into the range favorable for penicillin production (1). Thus, the efficiency of the mold for the production of penicillin is, in a very large measure, dependent on its biochemical characteristics; therefore, it would be of interest to investigate the biochemistry of penicillin formation in all its varied aspects.

Not much information appears to be available on the biochemistry

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of the mold, but the presence of vitamins and enzymes has been reported (3,4).

This paper presents the results of an investigation on the phosphatase in *Penicillium chrysogenum*, strain Q-176, which is reputed to be a very high yielder of penicillin and which is now almost exclusively used in the industry.

MATERIAL AND METHODS

Spores of *P. chrysogenum* Q-176 were sown on sporulating agar (5), and after about 8 days the fresh spores were scraped out and suspended in sterile water. This suspension was used for inoculating flasks containing the culture medium which had the following composition:

| | |
|--------------------------------------|----------|
| Corn steep liquor | 40 ml. |
| Lactose monohydrate | 27.5 g. |
| Glucose monohydrate | 3.0 g. |
| NaNO ₃ | 3.0 g. |
| MgSO ₄ ·7H ₂ O | 0.25 g. |
| KH ₂ PO ₄ | 0.50 g. |
| ZnSO ₄ ·7H ₂ O | 0.044 g. |
| MnSO ₄ ·4H ₂ O | 0.02 g. |
| Distilled water | 1 l. |

This culture medium was measured out in 100-ml. portions into 500-ml. flat-bottomed flasks and sterilized at 120°C. for 30 min., cooled, and then inoculated with the spore suspension. The high acidity of this medium was neutralized by the addition of about 1.0 g. of calcium carbonate per 100 ml. of medium, sterilized separately. By the third day after inoculation, there was visible growth on the surface of the medium and by the seventh day, there was a thick growth of mycelium which was then removed, dried superficially with blotting paper, ground into a fine paste on a glass mortar, and stirred into pure acetone. After an hour, the acetone was removed by filtration on the Buchner and the residue washed twice with acetone. It was then dried in a vacuum desiccator, powdered, and used as the source of crude enzyme.

Phosphatase activity was determined by measuring the inorganic phosphorus liberated by a weighed quantity of the acetone preparation suspended in 0.033 *M* veronal buffer or an aliquot of the extracts, using 1.0 ml. of a 10% solution of sodium β -glycerophosphate as the substrate. Inorganic phosphorus was determined by the colorimetric method described in the A. O. A. C. (6).

EXPERIMENTAL

Optimum Reaction for Enzyme Activity

The optimum reaction for the activity of the enzyme was determined by suspending 10 mg. of the acetone preparation in 5 ml. of veronal buffer adjusted to different pH's, and incubating the mixture at 25°C. for 3 hr. after the addition of 1 ml. of the substrate. The activity of the

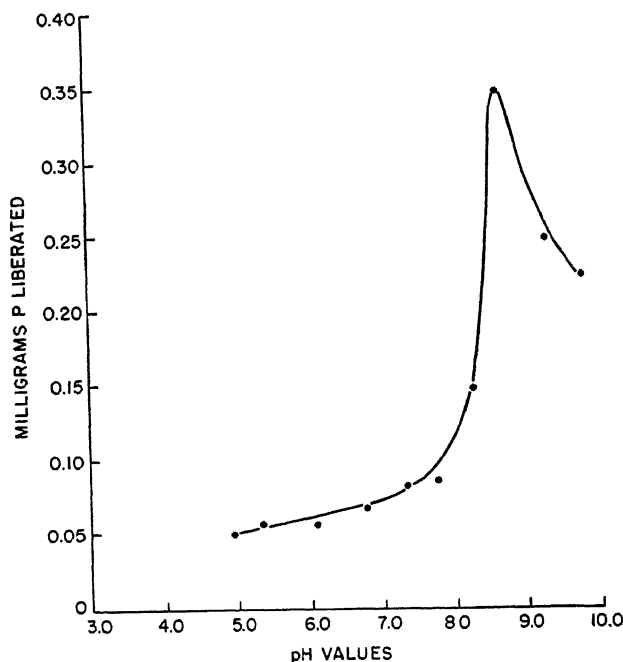


FIG. 1.

enzyme was arrested at the end of 3 hr. by the addition of 1 ml. of 1 *N* sulfuric acid, and the inorganic P determined colorimetrically. These results are presented in Fig. 1, from which it is seen that the optimum reaction for the activity of the enzyme centered about a pH of 8.7 and that there was no enzyme with a pH optimum on the acid side.

Rate of Extraction of the Enzyme

Weighed quantities of the acetone preparation (100 mg.) were placed in 50-ml. stoppered flasks and extracted with 25 ml. of water saturated with toluene for different lengths of time at the end of which the residue was separated by centrifugation. Aliquots of the supernatant (0.5 ml.) were used for the determination of enzyme activity and calculated for the original volume of water used. These results are presented in Table I from which it will be seen that extraction for a period of 24 hr. brings into solution about one-third of the total activity and that a second

TABLE I
Rate of Extraction of the Enzyme

| Period of extraction hr. | P liberated, calculated for the whole extract mg. |
|---|---|
| $\frac{1}{2}$ | 8.3 |
| 1 | 9.1 |
| 2 | 10.0 |
| $\frac{1}{2}$ | 10.3 |
| 6 | 12.1 |
| 24 | 13.0 |
| 2nd extraction (24 hr.) | 9.3 |
| In residue | 13.3 |
| Extraction of residue with glycerol (24 hr.) | 7.8 |

extraction releases almost an equal amount. There was still in the residue about a third of the total activity which could partly be extracted by shaking with 5 ml. of glycerol for 24 hr.

*Variation of Phosphatase Activity in Different
Strains of P. chrysogenum*

Numerous strains of the mold have been tried from time to time for the production of penicillin and it has been observed that the different strains have different capacities for the production of the antibiotic. Therefore, these strains were grown in corn steep liquor medium, described earlier, for a period of 7 days, the mycelium harvested, followed by treatment of the same with acetone three times in succession, dried, powdered, and kept in the desiccator. For the determination of

TABLE II
Variation of Phosphatase Activity in Different Strains of P. chrysogenum
(Four-day-old cultures dried after treatment with acetone; 10 mg.
of this powder used for the determination of activity)

| Strain | P liberated mg. |
|--------------------|--------------------|
| Q-176 | 0.300 |
| NRRL | 0.411 |
| N-22 | 0.500 |
| 832 | 0.732 |
| Westling and Brown | 0.602 |
| X-1612 | 0.503 |
| 1984 | 0.332 |

phosphatase activity, 10-mg. quantities of the powder were weighed out and suspended in veronal buffer adjusted to pH 8.7 with the addition of substrate. The inorganic phosphate was determined after 3 hr. incubation at 25°C. Table II shows the values for the different strains of *Penicillium* tested compared with Q-176. It is seen that the enzyme is present in all the strains tested though in varying concentrations as is to be expected.

Variation of Activity With the Temperature of Incubation

The acetone preparation from Q-176 was extracted twice with water saturated with toluene at the rate of 25 ml./100 mg. of the powder, and

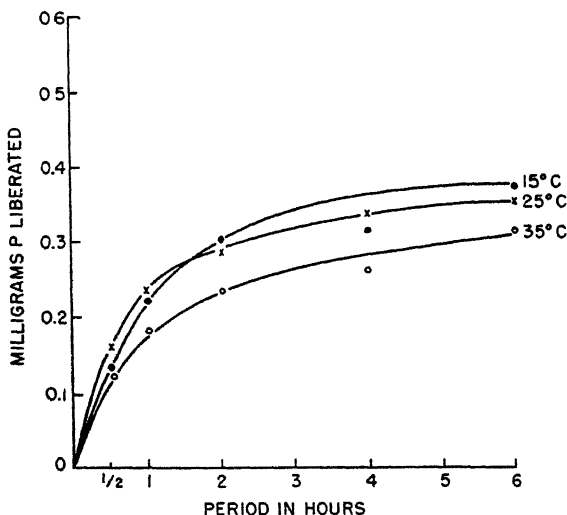


FIG. 2.

0.5 ml. of the aliquots of the clear centrifugate were used for all later investigations. The activity was determined at intervals of time at 15°, 25°, and 35°C., and the results are shown in Fig. 2. It is seen from these results that while there was not much difference in the activity of the enzyme when incubated at 15° and 25°C., there was an appreciable decrease in activity when the temperature of incubation was raised to 35°C. Therefore, all later experiments were carried out by incubation at 25°C.

Variation in Activity With Different Substrates

The activity of the enzyme on certain organic phosphates of physiological importance and on sodium pyrophosphate was tested. It was observed that the extract hydrolyzed phytin, nucleic acid (yeast), adenylic acid (yeast), fructose diphosphate, and sodium pyrophosphate (Table III).

Enzyme Activity and Age of Culture

It has been reported that during the growth of the mold, there is a rapid decrease in pH till about the 5th day in still cultures and till about 96 hr. in submerged cultures, and thereafter a rise in pH till the reaction of the metabolism fluid is about pH 8.0-8.5 (1,7). Since the optimum reaction for phosphatase activity is about 8.7, it is of interest to follow up the total activity of the enzyme in the mycelium and the metabolism fluid at different intervals of time during the period of growth of the mold. For this purpose, flasks containing 100 ml. of the medium were sown with equal volumes of a spore suspension and incu-

TABLE III
Activity of the Enzyme on Other Substrates

| Substrate | P liberated mg. |
|-----------------------------|--------------------|
| Phytin, 1% | 0.023 |
| Sodium pyrophosphate, 1% | 0.040 |
| Nucleic acid (yeast), 1% | 0.129 |
| Adenylic acid (yeast), 0.1% | 0.038 |
| Fructose diphosphate, 0.1% | 0.118 |

bated at 25°C. The mycelium was carefully removed by filtration through cotton wool at different intervals of time, and the corresponding metabolism fluid was also saved for the determination of enzyme activity. The mycelium was dried by treatment with acetone as described before, powdered, and 10-mg. samples used for the determination of activity, while 0.5 ml. of metabolism fluid was used for the same purpose. Necessary corrections were made for the inorganic phosphorus already present in the culture fluid by running controls without added substrate. The results are shown in Fig. 3 from which it is seen that the activity of the enzyme per unit weight of the mycelium and unit volume of the metabolism fluid increases with the age of culture, this increase being very much greater in the metabolism fluid, and that only after the 5th day.

Effect of Activators and Inhibitors on Enzyme Activity

The enzyme extract was precipitated with the addition of an equal volume of acetone, the precipitate centrifuged down and resuspended in 25 ml. of water, dialyzed against changes of distilled water in the cold (5°C.) for 48 hr. and this dialyzed preparation used for the studies on activation and inhibition. Among the compounds tried were salts of Mg, Mn, Ca, Zn, Co, oxalic acid, ascorbic acid, iodoacetic acid, HCN,

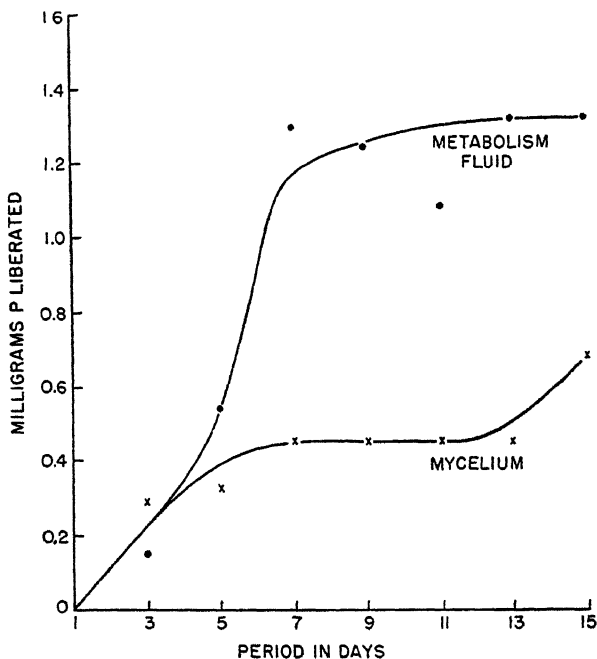


Fig. 3.

HF, sulfite, phlorizin, and phenylacetic acid. Phenylacetic acid was included because it has been found to increase the production of penicillin G in preference to other forms of penicillin, and it would be of interest to determine whether it has any other influence on general metabolic processes.

The present studies showed that additions of calcium and cyanide brought about inhibition of activity at 0.001 *M* and 0.002 *M*, respec-

tively, and that there was no appreciable difference in activity with the addition of other ions.

The inhibitory action of cyanide on the enzyme points to the possible presence of a metallic radical in it. Therefore, experiments were carried out with the enzyme treated previously with cyanide at a concentration of 0.002 *M* after neutralization of a pH of 8.7, and, thereafter,

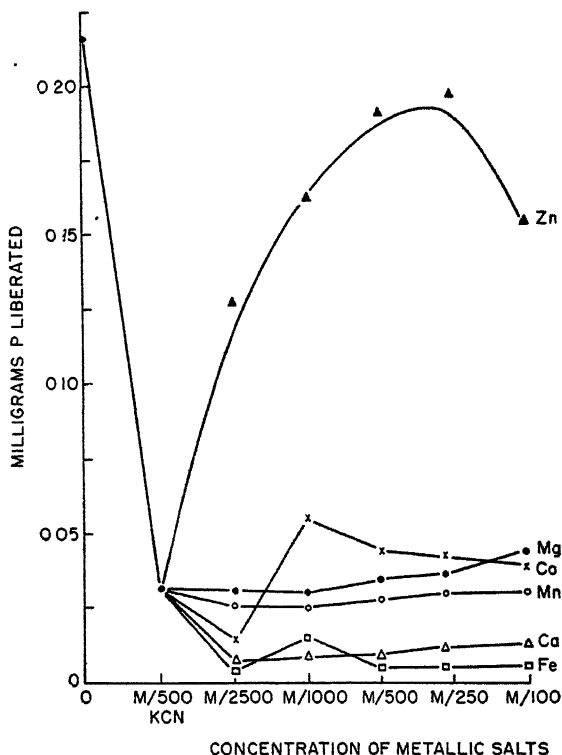


FIG. 4.

adding varying concentrations of different metallic ions. Regeneration of activity on the addition of any particular metallic ion would then throw light on the nature of the metal involved. These results are presented in Fig. 4 from which it is seen that only zinc ions had the power to regenerate the activity to an appreciable degree. It is also seen that higher concentrations of zinc lower the activity of the enzyme.

The reversal of cyanide inactivation on the addition of zinc was further investigated at different concentrations of the enzyme so as to see if, with increasing concentrations of the enzyme, proportionately increasing quantities of zinc are required. Figure 5 represents the observations of this experiment.

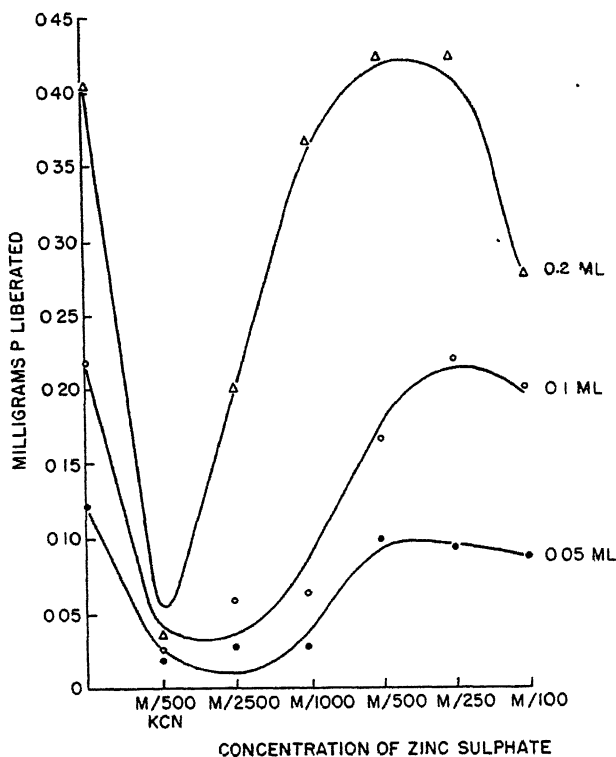


FIG. 5.

In another experiment, the activity of the cyanide-inactivated enzyme was compared with (a) enzyme untreated, (b) treated with cyanide at a concentration of $0.002\ M$ and then diluted with buffer to the same volume as in the other tubes, (c) inactivated by cyanide and then zinc at a concentration of $0.004\ M$ added before the addition of buffer, and (d) zinc added after the addition of buffer. This would show whether

TABLE IV
Reversal of Activity of Cyanide-Inactivated Enzyme by Zinc

| Treatment | P liberated mg. |
|---|--------------------|
| 1. Control, untreated (0.5 ml. of enzyme) | 0.198 |
| 2. Cyanide-inactivated enzyme (0.002 <i>M</i> HCN) | 0.025 |
| 3. As in 2. for 30 min. and then diluted to 10 ml. with buffer | 0.078 |
| 4. As in 2. and treated with zinc as ZnSO ₄ at 0.004 <i>M</i> followed by the addition of buffer | 0.168 |
| 5. As in 2. followed by dilution with buffer before the addition of zinc (0.004 <i>M</i>) | 0.151 |

the regeneration of activity of the enzyme is to be attributed to a lowering in the concentration of cyanide by precipitating it as zinc cyanide at the alkaline pH of the system, or by restoring the active ionic concentration of zinc in the enzyme complex. The results presented in Table IV show clearly that zinc acts directly on the enzyme itself.

DISCUSSION

The results presented in the foregoing pages indicate that the phosphatase in the mold is an alkaline enzyme which is inactivated by cyanide. The observation that the activity of the enzyme increases with the age of the culture and that it is highest at the stage when the pH of the metabolism fluid approaches the optimum reaction for the enzyme points to the fact that this enzyme is important in the metabolism of the mold, mainly in the third and last phase of its growth. This synchronizes with the period of maximum production of penicillin also. There is, likewise, a corresponding increase in the concentration of the enzyme in the metabolism fluid.

Of all the elements tested, zinc alone appears to have a marked influence on the activity of the enzyme. As was mentioned before, the enzyme is inactivated almost completely by cyanide and this inactivation is reversed only on the addition of zinc, which leads to the conclusion that the enzyme is probably a zinc complex. There appears to be an optimum concentration at which the element exercises its full activity. Higher concentrations are injurious to the enzyme. Calcium also appears to lower the activity of the enzyme, but the mechanism of its action is not clear.

ACKNOWLEDGMENTS

My thanks are due to Major-General Sir S. S. Sokhey and Dr. K. Ganapathi for their interest in the investigation.

SUMMARY

The properties of the phosphatase in *Penicillium chrysogenum* Q-176 have been studied. It is an enzyme with optimum reaction on the alkaline ranges of pH and is inhibited by cyanide at a concentration of 0.002 *M*. The inhibition is reversed on the subsequent addition of zinc sulfate at a concentration of 0.002 *M* to 0.004 *M*. Calcium also inactivates the enzyme at a concentration of 0.001 *M*. Evidence is adduced to show that it is a zinc-containing enzyme. The enzyme is elaborated by the mold at all the stages of its growth, but is more pronounced after the fifth day.

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Trace Element Impurities in Nutrient Solutions for Fungi

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INTRODUCTION

The writer has suggested at various times the desirability of improving upon the calcium carbonate method (10) for removal of trace element impurities from synthetic solutions used in micronutrient studies. A spectrographic check by Hughes (5) has revealed, in agreement with the results of nutrient studies, that all impurities are not removed with equal effectiveness.

It is true that various modifications in the method have been proposed by several investigators. None however appear to give a more complete removal of impurities. Hopkins (4) found it unnecessary to precipitate dicalcium phosphate in the nutrient solution with calcium carbonate. Direct addition of dicalcium phosphate to the clear solution appeared equally effective in removing manganese. Though Sakamura (8) used calcium phosphate in a similar manner for removal of micronutrient traces, the proposed modification is only an apparent one basically. The process of leaching employed probably resulted in the conversion of most of the calcium phosphate to dicalcium phosphate before use. Nevertheless, Sakamura appears to have demonstrated the effective action of dicalcium phosphate in nutrient solutions of moderate acidity.

The substitution of adsorbent carbon for calcium carbonate was proposed by Bortels (1). The objections to carbon are similar to those for calcium carbonate with the additional fault that the adsorbent introduces still other impurities: Roberg (7), Steinberg (10), Sakamura (8).

Extraction of the nutrient solution ingredients, singly or together, with organic solvents and dithizone have been used successfully in copper studies with green plants by Hoffman (3) and Piper (6). Hewitt,

Jones, and Nicholas (2) have found oxine precipitation of iron an effective method for removal of traces of molybdenum from culture solutions. Shu and Johnson (9) removed trace element impurities from sugar by precipitation of aluminum hydroxide with ammonia.

The results of a few of the better tests made by the writer over a long period in a search for a more effective process are presented in this paper. These studies included the use of modifications in the calcium carbonate method, the use of carbon and other adsorbents, hydrogen sulfide, dithizone, oxine, preculture on deficient solutions, and biological purification. The fungus, *Aspergillus niger* van Tiegh. was used as the test organism.

METHODS

A single spore culture of *A. niger* isolated initially in 1917 was used in these experiments. It was maintained on Czapek agar in stock cultures. This strain of the fungus is the only strain used in these and previous investigations and therefore permits of intercomparison of all experiments with a reasonable degree of accuracy. It is listed as No. 6275 in the American Type Culture Collection.

The fungus was grown as usual in 200-ml. Erlenmeyer flasks at a temperature of 35°C. for 4 days. All laboratory ware for micronutrient tests was of quartz. Distilled water was obtained by the condensation of building steam with a specially designed pyrex condenser system and stored in pyrex bottles. This water was twice distilled in quartz, condensed, and stored in quartz before use.

The nutrient solution used for purification contained per liter: sucrose, 50 g.; ammonium nitrate, 2.15 g.; dipotassium phosphate, 0.40 g.; and magnesium sulfate hydrate (7H₂O), 0.30 g. Purification was accomplished by adding 1 g. of calcium carbonate, or 60–80 mg. of spectroscopically pure calcium or magnesium oxide/l., autoclaving at 15 lb. for 30 min., and filtering through a No. 4 sintered quartz crucible. Fifty ml. of this solution was placed in each flask for culture of the fungus, and the appropriate micronutrients added. The micronutrient concentrations (in parts per million) were: iron, 0.6; zinc, 0.4; copper, 0.1; manganese, 0.2; molybdenum, 0.02; and gallium, 0.02. Both reagent and spectroscopically pure macronutrient salts were employed, but only spectroscopically pure chlorides of the micronutrients.

EXPERIMENTAL RESULTS

A summary of a few of the results in nutrient solution purification have been tabulated in Table I. The first experiment listed illustrates fairly well the degree of effectiveness of a single treatment with calcium carbonate on micronutrient removal using reagent chemicals. A second successive treatment gave still better results with all except iron. A single treatment with calcium oxide gave equally or improved results when all the salts used were spectroscopically pure (fourth experiment).

The next six trials that are listed also included the use of calcium oxide purification with spectroscopically pure materials. The sucrose used in the nutrient solutions was given various preliminary treatments to remove trace element impurities before treating the solution as a whole with calcium oxide. The procedure followed was to place the sucrose in a sintered pyrex glass thimble and to extract the sucrose with freshly distilled 95% alcohol or pyridine in a Soxhlet extraction apparatus. The sucrose gradually dissolved and crystallized out in the boiling

TABLE I
A Comparison of Some Procedures for Excluding Micronutrient Impurities in Deficiency Tests with A. niger

| Treatments (Inoculum of 62,500 spores/flask) | Initial pH | Control | Percentage yield on omission of | | | | | |
|---|---------------|---------|---------------------------------|-----|------|------|------|-------|
| | | | Fe | Zn | Cu | Mn | Mo | Ga |
| | | mg. | | | | | | |
| ^a Purified with CaCO ₃ | | | | | | | | |
| ^b Single treatment | 7.15 | 1142 | 0.4 | 4.0 | 84.3 | 42.1 | 81.1 | 96.1 |
| ^b Single treatment, 1/25 inoculum | 7.15 | 1111 | 0.3 | 0.8 | 70.4 | 33.3 | 74.9 | 84.4 |
| Double treatment | 7.18 | 1263 | 0.3 | 1.7 | 58.1 | 40.8 | 95.0 | 98.3 |
| Purified with CaO, special sucrose | | | | | | | | |
| control, unpurified sucrose | 7.40 | 1122 | 0.1 | 1.3 | 54.6 | 44.5 | 95.7 | 98.0 |
| Per pyridine | 7.42 | 992 | — | — | 44.5 | 18.3 | 66.0 | 105.0 |
| Per alcohol | 7.23 | 1118 | 0.5 | 0.2 | 36.9 | 23.8 | 58.8 | 93.6 |
| Per alcohol, 2 g. of carbon | 7.18 | 956 | 0.4 | 0 | 24.6 | 21.3 | 63.2 | 95.0 |
| Per alcohol, 2 g. of magnesium | 6.85 | 926 | 2.4 | 0.1 | 44.2 | 12.8 | 64.7 | 101.3 |
| Per alcohol, 4 g. of magnesium | 7.28 | 922 | 0.5 | 0 | 23.4 | 23.1 | 67.4 | 96.6 |
| ^b Biological purification | 6.65 | 1117 | 16.7 | 1.5 | 75.8 | 63.5 | 54.7 | 95.1 |
| ^b Minus Cu, Mo, and Ga, 18 transfers | 3.17 | 1073 | 4.8 | 4.4 | 84.5 | 19.1 | 59.4 | 72.8 |
| ^b Minus Cu, Mo, and Ga, 18 transfers | 7.20 | 858 | 2.2 | 8.6 | 97.1 | 22.6 | 67.8 | 81.6 |

^a Reagent chemicals. Other experiments with spectroscopically pure chemicals.

^b Average of two experiments.

flask. When no more sucrose remained in the extraction thimble the flask was removed, the solvent discarded, and the sucrose crystals dried. A layer of different adsorbent materials such as calcium carbonate, charcoal, powdered metallic magnesium, *etc.* was sometimes placed in the extraction thimble under the sucrose being treated.

Improved deficiency results were obtained in every case, except with iron, through preliminary purification of the sucrose. Pyridine gave results no better than those with alcohol. The use of under layers of

adsorbent charcoal or powdered metallic magnesium in the thimble gave appreciable improvements in removal of zinc, copper, and manganese. Biological purification, *i.e.*, use of a previously cultured nutrient solution gave an improvement in results only with molybdenum. A solution of double concentration to which no copper, molybdenum, or gallium had been added was inoculated with *A. niger* and filtered off after several days. It was then diluted to half concentration and again inoculated.

The last series of tests were concerned with the presence of micro-nutrients in the spores used for inoculating the deficiency test flasks ("carry-over"). It will be noted that culturing the fungus for eighteen transfers on a liquid medium containing a minimum of copper, molybdenum, and gallium gave improved results even without nutrient solution purification. Lower deficiency yields were thus obtained with zinc, manganese, molybdenum, and gallium.

✓ Another method of diminishing micronutrients unavoidably introduced into the solution with the inoculum is to decrease the size of the inoculum. A drop from 62,500 spores/flask to 2500 spores/flask led to appreciably greater decreases in yield on omission of iron, copper and manganese.

Other variations of the calcium carbonate method such as the use of calcium phosphate, magnesium phosphate, magnesium carbonate, or magnesium oxide appeared to give no better results with single treatments. A long series of tests was also carried out with other chemical ions than those of calcium and magnesium. The action of 10 p.p.m. of iron, aluminum, lanthanum, uranium, thorium, beryllium, lead, and arsenic as chloride was studied both alone and on simultaneous treatment of the neutral solution with calcium carbonate. In no case was an improvement in results observed. Aluminum, however, gave deficiency yields quite similar to those obtained through use of calcium carbonate. Evidently the precipitate of aluminum phosphate or hydroxide formed in the neutral nutrient solution was quite effective in adsorbing trace element impurities.

Precipitation with hydrogen sulfide at various acidities also led to no improvement in deficiency results except in one instance. At a pH of 2.83 and in the presence of bismuth ion (10 p.p.m.) a deficiency yield of 41.3% was obtained for molybdenum in the presence of ammonium nitrate as nitrogen source. This was appreciably better than the results obtainable with the calcium carbonate method. The writer (11) has

demonstrated the apparently greater requirement for molybdenum by *A. niger* with nitrate as compared to ammonium nitrogen. Molybdenum appears to participate particularly in the reduction of nitrate to ammonium. The bismuth sulfide coprecipitation method will probably be found adequate with nitrate nitrogen, therefore, for producing extreme molybdenum deficiencies with nutrient solutions containing sucrose.

Employment of dithizone either with organic solvents or in conjunction with calcium carbonate did not appear advantageous. Nor did the use of dithizone, oxine, quinalizarin, and several other organic substances prove useful in the extraction-recrystallization process with sucrose.

It might be assumed theoretically that complete absence of any micronutrient from the nutrient solution should give a zero yield in the absence of carryover in the inoculum. That this is an ideal condition that is usually never attained in practice is illustrated here by the effect of size and method of production of the inoculum material on yields. Complete absence of growth with deficiency was obtained only twice, and both times with zinc. A combination of methods will in all probability be necessary to obtain maximum results. The combination would include inocula of minimum size and micronutrient content and nutrient solutions exhaustively purified to remove trace element impurities.

Purification with calcium carbonate should be repeated until no further improvement in results are to be anticipated. The number of successive treatments will depend on the quantities of impurities originally present. A preliminary treatment of the sucrose with either adsorbent charcoal or magnesium metal powder is also helpful in obtaining maximum purity quickly.

Several factors should be taken into account in comparing the results obtained with various methods of purifying the nutrient solution. Fungous studies are with nutrient solutions containing sugar, which retains minute impurities tenaciously. Fungi also appear to require appreciably smaller quantities of micronutrients than do green plants. Hoffman (3) and others have found dithizone extraction satisfactory for green plants. The writer has at no time been able to make successful use of the dithizone method as compared with the calcium carbonate method.

The writer had previously estimated that a purity of one part per billion would be necessary in order to identify all the micronutrients necessary for microorganisms. Present levels of purity are probably more than ten times lower. These impurities presumably affect the

results obtainable with the known essential elements, and may therefore lead to erroneous results and interpretations.

SUMMARY

Improved results in micronutrient deficiency studies required a combination of procedures including preculture of the fungus on deficient solutions and use of minimum quantities of inoculum. Maximum removal of trace element impurities from the medium for *Aspergillus niger* van Tiegh. required the use of the purest available compounds. The number of successive treatments with calcium carbonate for effective trace element removal depended on the quantity of impurities initially present. A preliminary treatment of the sucrose with adsorbent carbon or powdered metallic magnesium led to improved trace element removal. The combination procedure should give deficiency yields (in per cent of maximum growth) less than or equal to the following: iron, 0.1; zinc, 0; copper, 23.4; manganese, 12.8; molybdenum (ammonium nitrate), 57.4; and gallium, 70.7. The substitution of aluminum chloride for calcium carbonate in a neutral nutrient solution and filtration also gave equally satisfactory removal of trace element impurities. Coprecipitation with bismuth sulfide gave improved removal of molybdenum with media containing sucrose.

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The Metabolism of Stem Tissue During Growth and Its Inhibition. III. Nitrogen Metabolism¹

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INTRODUCTION

The metabolism of carbohydrate and ether-soluble materials in isolated sections of *Pisum* internodes has been described in previous papers of this series (2,3). Promotion of growth (elongation) by auxin was shown to be correlated with an increase of oxidative metabolism (Q_{O_2}), but inhibition of growth was not necessarily accompanied by a decrease in oxidation rate. Also it was shown that the increased oxidation accompanying growth does not involve an additional destruction of sugar but rather takes place at the expense of fats. Respiratory quotient (R. Q.) measurements corroborated these conclusions based on chemical analyses of the tissues. Additional changes were found to take place in the sucrose content and in the constituents of the cell wall. It follows that the effects of auxin and of the three growth inhibitors used in this study (iodoacetate, arsenite, and fluoride) are very deep-seated. It was therefore considered necessary to continue the study of the changes in metabolites which accompany growth and inhibition. The present paper reports the changes in nitrogenous substances, including free ammonia, amides, amino acids, and protein. The idea, previously expressed, that *all* metabolic systems of this tissue are fundamentally modified by auxin and by the inhibitors is borne out by these experiments.

EXPERIMENTAL

The general methods of growing plants, cutting 20-mm. sections, and preparation of growth solutions have already been described (2). The

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sections were examined when initially cut from the plants, and after 24 hr. in five different solutions, namely, water, auxin (indole acetic acid 1 mg./l.), auxin plus iodoacetate $6 \times 10^{-4} M$, auxin plus arsenite $10^{-4} M$, and auxin plus fluoride $5 \times 10^{-3} M$. These concentrations of the inhibitors reduced growth to 50% of that of the auxin controls. The present experiments include, first, determinations of total nitrogen by the micro-Kjeldahl method. This total nitrogen was subdivided into protein and non-protein nitrogen by precipitation with trichloroacetic acid. The cell-wall protein had previously been determined with the other cell-wall constituents (2) and the remainder of the insoluble nitrogen was considered to be in the form of plasma protein. The soluble nitrogen was further subdivided by determinations of free ammonia and amides, the remainder being considered to be principally free amino acids. This fraction also contains free bases (12) and small amounts of purines and peptides. However, for the present purposes determinations of these minor constituents were not necessary.

Amides

Because of the known occurrence of asparagine and glutamine in large amounts in etiolated legume seedlings (1,9) these amides were determined separately, using the method of Vickery and Pucher (13) and the Pucher vacuum still (8). Samples of 30 sections each were grown for the 24-hr. period in the various growth solutions, then were ground with sand at 4°C. to a fine paste, which was then extracted five times with cold water.

The method determines the free ammonia (or ammonium ion), ammonia plus the amide of glutamine, and ammonia plus the amide of both glutamine and asparagine. This is done by making one aliquot mildly basic, hydrolyzing another under conditions that have no effect on asparagine, and hydrolyzing a third completely. After each treatment the ammonia is distilled off under vacuum.

Free Amino Acids

The non-protein nitrogen (NPN) was determined by the method of Steward and Preston (10) using trichloroacetic acid as protein precipitant. Samples of 20 sections each were ground with sand immediately after the growth period, the resulting pulp was covered with 2.5% trichloroacetic acid and the mixture was stirred at room temperature for 15 min., then filtered into 30 ml. Kjeldahl flasks. The filtrate was evaporated nearly to dryness, and digested with concentrated H_2SO_4 and H_2O_2 . The nitrogen was then determined by the micro-Kjeldahl method according to Pregl (7). The nitrogen of the amides and ammonia was subtracted from these NPN values. For the purposes of studying the interconversion of bulk metabolites, the presence of small amounts of bases, *etc.*, is of minor importance; the remaining nitrogen was therefore

considered as amino acids, and was multiplied by the factor 6.2 to give the quantity of free amino acids.

Protein

Total nitrogen was determined on samples of 15 sections each. At the end of the 24-hr. growth period, the whole sections were transferred to 30-ml. Kjeldahl flasks and incinerated with H_2SO_4 and H_2O_2 and the nitrogen determined by the micro-Kjeldahl procedure (7). From these total nitrogen values were subtracted the NPN values, and the differences multiplied by the factor 6.2 to obtain the total protein content of the sections. This was corrected for the cell-wall protein which had been determined previously (2) and the difference was considered as plasma protein.

RESULTS

Amides

The results of the amide determinations are presented in Table I. It is seen that the sections contained a very small amount of free ammonia

TABLE I
Free Ammonia and Amides in Pea Stem Sections
(Per cent of initial dry weight) ^a

| Treatment | Total nitrogen of amide compounds | Amide compound | | |
|----------------------------------|---|----------------|-----------|------------|
| | | Ammonia | Glutamine | Asparagine |
| Initial | 2.11 | 0.11 | 3.17 | 6.69 |
| Water control | 2.73 | 0.19 | 1.20 | 11.03 |
| Auxin (1 mg./l. IA) | 2.83 | 0.16 | 1.36 | 11.48 |
| Auxin plus: | | | | |
| iodoacetate $6 \times 10^{-4} M$ | 2.10 | 0.16 | 1.29 | 8.12 |
| arsenite $10^{-4} M$ | 2.05 | 0.26 | 1.21 | 7.53 |
| fluoride $5 \times 10^{-3} M$ | 2.11 | 0.29 | 1.36 | 7.67 |

^a Average of duplicate determinations on duplicate tissue samples.

initially and that this amount increased slightly during growth under all conditions, the presence of arsenite and fluoride perhaps causing a slightly greater formation of ammonia, though the difference is probably not significant. The glutamine content decreased in all cases as a function of time but not of treatment. Asparagine, however, shows striking changes. During growth in auxin the asparagine content was very nearly doubled. In water the increase was only slightly less. All three inhibitors, on the other hand, largely prevent this rise.

Amino Acids

As stated above, the non-protein nitrogen corrected for amides and ammonia is regarded here as free amino acids. The data given in Table II show that growth in auxin is accompanied by a large decrease in this free amino acid fraction, amounting to virtually complete disappearance. In water there is not quite so great a loss, while all three inhibitors oppose this transformation. The differences between the effects of the inhibitors are small and it is evident that the action of all three on amino acid consumption is essentially the same. Arsenite appears to be slightly more effective than fluoride, and iodoacetate slightly less. The low figure for amino acid content of iodoacetate-treated sections is probably a reflection of the loss of nitrogenous substances to the growth solution [see Table V and Ref. (4)].

TABLE II
Non-Protein Nitrogen and Free Amino Acids^a of Pea Stem Sections
(Per cent of initial dry weight)

| Treatment | NPN ^b | NPN - amide N = nitrogen of amino acids ^c | Amino acids ^d |
|----------------------------------|------------------|--|--------------------------|
| Initial | 4.34 | 2.23 | 13.7 |
| Water control | 3.04 | 0.31 | 1.9 |
| Auxin (1 mg./l. IA) | 2.93 | 0.10 | 0.6 |
| Auxin plus: | | | |
| iodoacetate 6×10^{-4} M | 3.20 | 1.10 | 6.8 |
| arsenite 10^{-4} M | 3.46 | 1.41 | 8.7 |
| fluoride 5×10^{-3} M | 3.31 | 1.20 | 7.5 |

^a Amino acids includes also free purines and natural bases.

^b Non-protein nitrogen; average of duplicate determinations on duplicate tissue samples.

^c Amide N is total N of amide compounds and ammonia from Table I.

^d Calculated from column 2 by multiplying by factor 6.2.

Extracts (deproteinized as above) of the sections before and after the various treatments were qualitatively analyzed for amino acids by paper chromatography. The methods used ³ were patterned after those of Dent (5,6) except that the spots were spread out by ascending solvents rather than descending (14), and the ninhydrin color was developed by

³ The authors desire to thank Prof. F. C. Steward for his advice and suggestions, and for subsequent careful criticism of the manuscript.

heating with an electric iron. The first modification is claimed to have the advantage that a more even solvent front is obtained; the second modification allows control of the color development and enables individual detection of two spots very close together which would otherwise appear as a single spot. The phenol used was redistilled under vacuum from zinc dust and did not darken in use; for this reason cyanide was not used in the cabinet; the aqueous phenol had a pH of 3.8; ammonia was also not used in the cabinet.

Figure 1 shows the position taken up by the various amino acids present in the extracts of the sections. The spots are numbered as in Dent's diagram (5). Comparison of the two "maps" shows that the exact positions are not identical but that the relative pattern is the same. The spots, with the exception of those ascribed to α -amino-n-butyric acid (No. 26) and hydroxylysine (No. 31), were identified by close comparison with chromatograms of known mixtures run *simultaneously* with the chromatogram of tissue extracts. Samples of these two acids were not available and hence their identification is not absolute and depends only on the appearance of spots in the relative positions established by Dent. Four of the spots designated by letters in Fig. 1 are due to unidentified peptides as shown by their disappearance on acid hydrolysis.

In view of the relatively large quantities of stachydrin and choline isolated from leaves by Vickery (12), an attempt was made to identify these compounds in our extracts.⁴ However, their ninhydrin reactions after chromatographing were too weak to allow of their detection. Attention is also called to the fact that no spot for proline was found, but a spot for hydroxyproline, identical both in position and in its characteristic yellow color with those in the reference chromatogram, was found consistently; according to Steward (11) this occurs rarely in plant extracts. The absence of detectable amounts of cysteine and cystine (or cysteic acid, to which these amino acids are converted during chromatography) is notable. This observation is confirmed by the nonappearance of a cysteic acid spot after treatment with H_2O_2 .

The amino acids which are present in the extracts, and the relative intensities of the spots, are shown in Table III. A designation of 1 indicates a just visible spot while 5 represents that spot having the greatest intensity, namely, asparagine in each case. These relative intensities can be taken only as a rough indication of the relative amounts of the

⁴ A sample of stachydrin was generously supplied by Dr. H. B. Vickery.

TABLE III
Free Amino Acids in Water Extracts of Pea Stem Sections

| Spot no. ^b | Substance | Relative intensity of spot compared to that of asparagine ^a | | | | | |
|-----------------------|---|--|-------|-------|--|------------------------------------|---|
| | | Treatment | | | | | |
| | | Initial | Water | Auxin | Auxin + $6 \times 10^{-4} M$ iodoacetate | Auxin + $10^{-3} M$ arsenite | Auxin + $5 \times 10^{-3} M$ fluoride |
| 5 | Aspartic acid | 0-1 | 2 | 2 | 2 | 3 | 3 |
| 6 | Glutamic acid | 2 | 2 | 2 | 2 | 2 | 3 |
| 9 | Serine | 2 | 1 | 1 | 1 | 2 | 2 |
| 12 | Glycine | 3 | 2 | 2 | 3 | 3 | 3 |
| 13 | Asparagine | 5 | 5 | 5 | 5 | 5 | 5 |
| 16 | Threonine | 4 | 3 | 3 | 3 | 3 | 3 |
| 18 | Tyrosine | 2 | 2 | 2 | 2 | 2 | 2 |
| 20 | α -Alanine | 4 | 4 | 4 | 4 | 4 | 4 |
| 21 | Glutamine | 3 | 3 | 3 | 3 | 2 | 3 |
| 26 | α -Amino- <i>n</i> -butyric acid | | | | | | |
| | (?) | 3 | 3 | 3 | 3 | 3 | 2 |
| 27 | Histidine | 4 | 3 | 4 | 4 | 4 | 3 |
| 28 | Hydroxyproline | 2 | 1 | 2 | 2 | 1 | 0 |
| 29 | β -Alanine | 1 | 3 | 3 | 2 | 2 | 2 |
| 31 | Hydroxylysine (?) | 1 | 2 | 0 | 0 | 3 | 1 |
| 32 | Tryptophan | 3 | 3 | 4 | 3 | 3 | 4 |
| 34 | Methionine ^d | 3 | 2 | 2 | 3 | 3 | 3 |
| 35 | Norvaline ^e | 3 | 2 | 1 | 3 | 1 | 2 |
| 36 | Valine | 4 | 3 | 3 | 4 | 4 | 4 |
| 41 | Ornithine | 2 | 2 | 2 | 2 | 2 | 2 |
| 44 | Phenylalanine | 2 | 2 | 2 | 4 | 2 | 2 |
| 45 | Leucine | 4 | 3 | 3 | 3 | 2 | 2 |
| 46 | Isoleucine | 2 | 3 | 2 | 4 | 3 | 2 |
| 56 | Arginine | 4 | 2 | 2 | 3 | 3 | 4 |
| 58 | Lysine | 3 | 2 | 2 | 2 | 2 | 2 |
| A | Peptide | 2 | 0 | 2 | 3 | 2 | 2 |
| B | Probably methionine sulfoxide ^d | 3 | 2 | 3 | 3 | 3 | 2 |
| C | Peptide | 3 | 2 | 0 | 0 | 0 | 3 |
| D | Peptide | 2 | 2 | 0 | 0 | 2 | 0 |
| E | Peptides | 3 | 2 | 3 | 2 | 3 | 3 |

^a Relative intensities of the spots developed by ninhydrin were estimated and designated by number from 1, spots just visible, to 5, the spot of greatest intensity, asparagine in each case.

^b The numbering of the spots corresponds to Dent's system (5). These numbers identify the spots of Fig. 1.

amino acids. Furthermore, it should be pointed out that the amount of extract used in the chromatograms for the sections grown in water and auxin was about eight times that for the other treatments. Thus the great decrease in amino acids that takes place during growth was more or less compensated and spots of satisfactory intensity obtained.

TABLE IV
Total Nitrogen and Protein Components of Pea Stem Sections
(Per cent of initial dry weight)

| Treatment | Total N ^a | NPN ^b | Difference = protein nitrogen | Calculated Total protein ^c | Cell- wall ^d protein | Plasma protein |
|----------------------------------|----------------------|------------------|-------------------------------------|---|---------------------------------------|-------------------|
| Initial | 7.03 | 4.34 | 2.69 | 16.7 | 3.4 | 13.3 |
| Water control | 6.80 | 3.04 | 3.76 | 23.3 | 4.4 | 18.9 |
| Auxin (1 mg./l. IA) | 6.88 | 2.93 | 3.94 | 24.4 | 4.3 | 20.1 |
| Auxin plus: | | | | | | |
| iodoacetate 6×10^{-4} M | 5.98 | 3.20 | 2.78 | 17.2 | 3.8 | 13.4 |
| arsenite 10^{-4} M | 6.61 | 3.46 | 3.15 | 19.5 | 4.2 | 15.3 |
| fluoride 5×10^{-3} M | 6.63 | 3.31 | 3.32 | 20.7 | 4.1 | 16.6 |

^a Average of duplicate determinations on duplicate tissue samples.

^b From Table II.

^c From N values by multiplying by factor 6.2.

^d From Table VIII of Ref. (2).

It is apparent from Table III that the differences in amino acid composition which accompany growth and the inhibition of growth are entirely quantitative. The behavior of valine and arginine is perhaps worthy of comment, since these two amino acids appear to decrease somewhat more than the average during growth and to be prevented

^e The identification of the spots was made by close comparison with simultaneous chromatograms of mixtures of known amino acids except for No. 26 (α -amino-*n*-butyric acid) and No. 31 (hydroxylysine).

^f The presence of methionine was proved by H_2O_2 treatment which converts methionine to methionine sulfone, spot No. 25. The spot marked "B" is probably methionine sulfoxide (No. 39 in Dent's map) which is formed from methionine during the phenol run. It is not excluded, however, that "B" is due to γ -aminobutyric acid (11).

^g The identification of norvaline depends on the clear appearance of a spot in position 35 (separate from number 36) during development of the ninhydrin color, as well as on comparison with a simultaneous chromatogram of the pure substances. In view of the rarity of norvaline in plant tissues, it is well to point out that the simultaneous presence of number 36 (valine) makes the identification more certain.

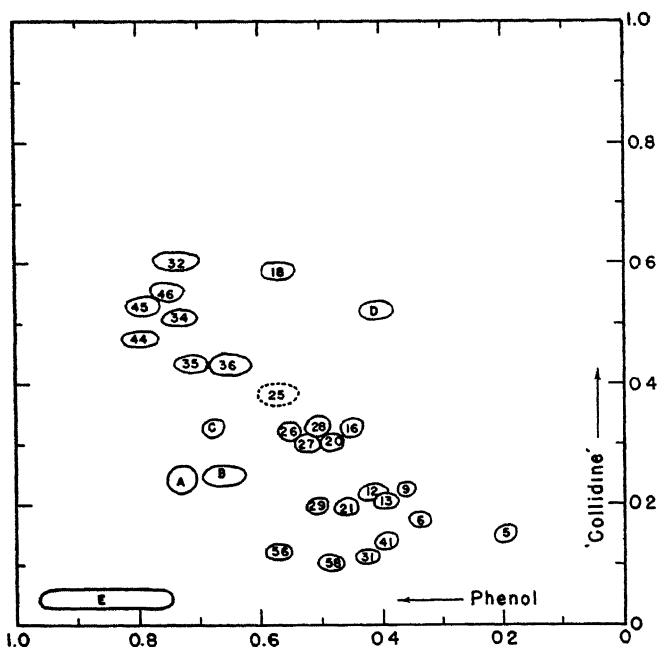


FIG. 1. Scale "map" of the spots of ninhydrin-reacting substances of water extracts of pea stem sections. The amino acids start at the lower right-hand corner and are spread out by phenol-water passing first from right to left and then "collidine" (1:1 mixture of 2,4-lutidine and 2,4,6-collidine) (6) passing vertically to the extreme edges. The scales represent R_F values. The numbering of the spots is the same as in the "map" published by Dent (5) and the numbered spots are identified in Table III.

from decreasing when growth is inhibited. Aspartic acid shows the opposite behavior, since it is present in barely detectable traces in the fresh tissue, but increases after growth. It may also be noted that, next to asparagine, alanine appears to be the most abundant amino acid in all cases.

Protein

Table IV presents the data on protein content. It is seen that there is considerable synthesis of protein during growth in auxin, and only slightly less in water. When growth is inhibited 50% by iodoacetate, protein synthesis is almost entirely prevented. The 50%-inhibiting concentrations of arsenite and fluoride act in qualitatively the same way but are not quite so effective in preventing protein synthesis. In general,

the failure to synthesize proteins very closely parallels the reduced consumption of amino acids shown in Table II.

DISCUSSION

The changes of all nitrogenous substances after the various treatments are brought together in Table V expressed as nitrogen content. In Table VI the content of the important constituents (expressed as percentage of dry weight) is summarized. The point of greatest interest in these tables is the clear demonstration that the principal process of nitrogen metabolism in this tissue is the very rapid conversion of free amino acids to protein and to asparagine. The amino acid nitrogen which disappears during growth becomes about equally divided between

TABLE V
Changes in Nitrogenous Substances Expressed as Nitrogen Content
(Per cent of initial dry weight^a)

| Treatment | Ammonia | Glutamine | Asparagine | Amino acid | Protein | | | Total nitrogen |
|----------------------------------|---------|-----------|------------|------------|-----------|-----------|-------|----------------|
| | | | | | Cell wall | Cytoplasm | Total | |
| Water control | +0.07 | -0.38 | +0.92 | -1.90 | +0.16 | +0.90 | +1.06 | -0.23 |
| Auxin (1 mg./l. IA) | +0.04 | -0.35 | +1.01 | -2.11 | +0.14 | +1.10 | +1.24 | -0.17 |
| Auxin plus: | | | | | | | | |
| iodoacetate $6 \times 10^{-4} M$ | +0.04 | -0.36 | +0.30 | -1.11 | +0.06 | +0.02 | +0.08 | -1.05 |
| arsenite $10^{-4} M$ | +0.13 | -0.38 | +0.19 | -0.81 | +0.13 | +0.32 | +0.45 | -0.42 |
| fluoride $5 \times 10^{-4} M$ | +0.15 | -0.34 | +0.22 | -0.99 | +0.11 | +0.53 | +0.64 | -0.40 |

^a Figures in this table are the difference between analyses of initial samples at time of cutting and analyses after 24 hr. growth in solution.

TABLE VI
Content of Principal Nitrogen Compounds Before and After Growth and Inhibition
(Per cent of initial dry weight)

| Treatment | Elongation | Amino acid | Protein | Asparagine |
|----------------------------------|-----------------|------------|---------|------------|
| | <i>per cent</i> | | | |
| Initial | — | 13.7 | 16.7 | 6.7 |
| Water control | 20.0 | 1.9 | 23.3 | 11.0 |
| Auxin (1 mg./l. IA) | 50.9 | 0.6 | 24.4 | 11.5 |
| Auxin plus: | | | | |
| iodoacetate $6 \times 10^{-4} M$ | 25.6 | 6.8 | 17.2 | 8.1 |
| arsenite $10^{-4} M$ | 26.5 | 8.7 | 19.5 | 7.6 |
| fluoride $5 \times 10^{-4} M$ | 25.3 | 7.5 | 20.7 | 7.7 |

these two forms. In auxin this process is stimulated by slightly over 10%. The three inhibitors, in concentrations which reduce the growth of the sections to 50% of that taking place in auxin, correspondingly inhibit the consumption of amino acids by about 50% (48%, 61% and 53%, respectively). Along with this, the formation of asparagine and the synthesis of proteins are inhibited to about the same extent, *i.e.*, about 50%, more or less. Such a correlation of the synthesis of proteins with the formation of asparagine and with growth suggests very strongly that these transformations of nitrogenous substances are closely interdependent and *further*, that the whole process is critical for growth. It is tempting to propose a scheme whereby the formation of a peptide bond is necessarily accompanied by the formation of one molecule of asparagine, but such a scheme would be at present entirely hypothetical.

This is, we believe, the first demonstration that growth by cell elongation is accompanied by protein synthesis. However, Steward and Preston (10) demonstrated the synthesis of protein during the uptake of water by potato slices, a process which certainly has much in common with growth.

Consideration of the data on total nitrogen (Table V) shows that a small amount of nitrogen is lost from the sections in all treatments. This loss may be either through production of ammonia (or other volatile product) or through exosmosis of nonvolatile nitrogenous solutes. Nesslerization of the solutions after completion of growth showed no detectable ammonia. However, determinations of dry weights of the growth solutions showed that in the iodoacetate-inhibited sections there was a considerable exosmosis of solid material (4). The analysis of this exudate will be discussed in detail in a subsequent paper, but for present purposes it is sufficient to mention that, of the 1.05% of the initial dry weight of nitrogen lost by the iodoacetate-treated sections, 0.84% is present as a mixture of amino acids, in the nonvolatile exudate. This figure accounts for most of the difference between iodoacetate and the other two inhibitors, since with them the exosmosis was barely detectable.

The data show clearly that in this tissue amides are not formed as a result of proteolysis, but on the contrary are associated with protein *synthesis*. This observation parallels the conclusion of Wood *et al.* (15) who showed that amide formation in grasses took place under aerobic and not under anaerobic conditions though proteolysis occurred in both cases. In the recent extensive experiments of Yemm (16) in starving barley leaves it is established still more clearly that the asparagine

formed cannot arise from 4-carbon residues in the protein, since the total yield after about 100 hr. of proteolysis is much greater than the sum of aspartic acid and asparagine present in the initial protein. If, then, asparagine is formed *de novo*, there is no particular reason to associate it with proteolysis, and indeed, it may even be suggested that amides are formed in association with protein synthesis, as a general rule. In the classical case of the lupine seedling (1), amide formation has been associated with proteolysis because attention was focused on the proteolysis within the seed. But actually protein synthesis in the developing seedling is at least equally important and it is in the seedling, *not* in the cotyledons, that most of the asparagine is found.

The large amounts of asparagine formed during growth require a similarly large consumption of 4-carbon organic acids. However, as was shown previously (3), the net change in organic acids during growth is small. It is quite probable, then, that this drain of Krebs cycle acids is made up, or nearly so, by the oxidation either of sugars or of the deaminated residues of amino acids. Hence, the slight over-all decrease in organic acids has little or no relationship to the amount of asparagine formed.

It is very probable, however, that compounds having the same C4 skeleton as asparagine play a dynamic central role in the processes involving nitrogenous metabolites, as well as in the metabolism of fats and carbohydrates. Thus it has now been shown that the action of auxin and of the three inhibitors on growth involves the metabolism of sugars, fats, amino acids, asparagine, and protein. The point at which all these processes come together is in the metabolism of the 4-carbon acids, and it must be here that the inhibitors exert their action on growth. The effect of such action on nitrogenous metabolism would be through oxalacetic acid, which would be made less "available" by the inhibitors. Since no appreciable amount of ammonia is formed, it follows that deamination of amino acids is dependent on the availability of such ammonia acceptors as oxalacetic and aspartic acids, and hence changes in the availability of these compounds would have a parallel effect on the rate of amino acid destruction. And since this destructive process appears to be a necessary adjunct of protein synthesis, the same control of organic acid metabolism would control protein synthesis.

The action of auxin cannot be exerted simply in the opposite direction to that of the inhibitors. Its role must be to "link up" in some way the metabolic processes discussed above to the phenomena which result

in the uptake of water, that is, visible growth. This is probably accomplished through a metabolic system closely related to those on which the inhibitors act. Such a linkage does not involve a greatly increased consumption of metabolites but may be thought of as making the metabolism functionally effective.

SUMMARY

The nitrogen metabolism of isolated pea stem sections and the influence of indoleacetic acid and three enzyme inhibitors (arsenite, fluoride, and iodoacetate) on this metabolism have been studied.

The principal changes taking place when sections are maintained in water are: (1) Consumption of free amino acids, (2) synthesis of protein, and (3) formation of asparagine. These three processes proceed at such relative rates that the nitrogen of the amino acids consumed is almost equally divided between the protein and asparagine formed.

These changes are stimulated by indoleacetic acid in a concentration which optimally promotes growth, and are inhibited about 50% by the three inhibitors in concentrations which inhibit growth by 50%. It is concluded that the above process is one of several which are critical for growth.

Extracts of the sections were qualitatively analyzed for free amino acids by paper chromatography. It was found that 29 ninhydrin-reacting substances are present initially, asparagine and alanine being the most important; all of these decrease markedly during growth. The changes in content and their possible relation to growth are discussed.

Changes in other nitrogenous materials, including free ammonia, glutamine, and cell-wall protein are slight and show less variation with treatment.

It has been demonstrated that growth by cell enlargement is accompanied by protein synthesis.

The effects of auxin and the three inhibitors on these processes of nitrogen metabolism and on the carbohydrate and fat metabolism previously described are explained by postulating that their influence is mediated through the metabolism of the 4-carbon acids.

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The Metabolism of Stem Tissue During Growth and Its Inhibition. IV. Growth Inhibition without Enzyme Poisoning¹

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INTRODUCTION

It has been reported in parts I, II and III of this series (3,4) that when isolated sections of the stems of pea seedlings are allowed to elongate in auxin solution certain metabolic changes take place. These are in the same direction as when the sections are simply maintained in water, but are quantitatively accentuated. Reducing sugars and fats are consumed, while cell-wall polysaccharides are laid down. Amino acids also are consumed, and proteins are formed, together with a considerable amount of asparagine. The respiration is increased some 15% by the auxin, and the respiratory quotient (R. Q.) is lowered from a value slightly above 1 to about 0.9.

When growth is inhibited, large changes in metabolism occur. The content of reducing sugars is lowered, while that of fats is increased; the over-all oxygen uptake may be little changed, but the R. Q. is definitely raised. The formation of asparagine is also strongly inhibited. Three different growth inhibitors, namely iodoacetate, arsenite and, fluoride, produce essentially similar effects. In view of the fact that the enzymatic basis for the inhibition of growth by fluoride is presumably entirely different from that involving iodoacetate and arsenite, this result is somewhat surprising. It indicates either (α) that growth is controlled by a number of different processes, interference with any one

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of which may bring about essentially the same chain of biochemical results, or else (b) that the metabolic changes referred to are the result, rather than the cause, of the growth inhibition.

This paper is concerned with the second alternative. *A priori*, it is by no means improbable that interference with growth might secondarily result in characteristic changes in metabolism. Against this is the fact that although the metabolic effects of the three inhibitors mentioned are similar, they are not identical, and when growth is inhibited to the same degree (*i.e.*, 50%) by each of them, there are clear-cut, though minor, differences in the metabolic pattern. For further analysis of the action of inhibitors on growth, it is essential to know whether the changed metabolism which accompanies inhibition is a direct or indirect result of their action.

Fortunately the problem can be settled directly, for growth can be inhibited by purely physical means without involving the poisoning of an enzyme. This follows from the fact that the growth of these isolated sections in simple solutions can essentially be regarded as the metabolically controlled uptake of water. Now it was shown more than ten years ago (7) that the growth of coleoptile sections in auxin solution could be controlled through the osmotic gradient. By adding mannitol to the auxin solution the extent of growth was shown to be in linear inverse proportion to the external osmotic concentration. At a 0.31 *M* concentration, growth was zero.

It was decided therefore to follow certain of the principal metabolic changes when growth was inhibited osmotically. Reducing sugars and amides were selected as being most characteristic. Mannitol was unsuitable for part of the present experiments because of the possibility that it might be converted to reducing sugars. Recourse was therefore had to inorganic salts which, in sufficiently high concentration, will markedly inhibit growth. For the experiments on nitrogen metabolism, use was made of both mannitol and inorganic salts.

EXPERIMENTAL

Effects of Salts on Growth of Pea Stem Sections

Sections 20 mm. long were cut from the apex of the third internode of etiolated pea seedlings as previously described (3) and their growth in solutions measured after 24 hr. in darkness. Indoleacetic acid at 1 or 10 mg./l. was added to all solutions unless otherwise noted.

Potassium chloride occupies a special place since it promotes growth at concentrations up to $0.01\ M$ both as straight growth (3) and in the curved growth of the pea test (6); a similar effect was shown much earlier in our studies on coleoptile sections (7). In numerous experiments with pea stem sections KCl was found to cause very slight promotion of elongation, the optimum concentration being at $0.03\ M$. The promotion is not as large as that caused in the presence of sucrose [see Ref. (3), Tables I and II]. Inhibition begins at about $0.07\ M$, but even $0.1\ M$ KCl causes only about 13% inhibition.

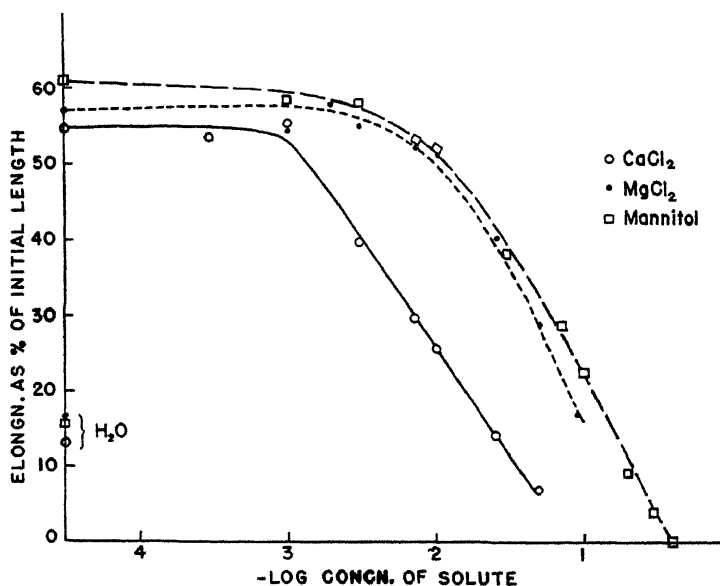


FIG. 1. The elongation of 20-mm. apical sections of etiolated pea stems in indoleacetic acid, 10 mg./l., with the addition of solutes as shown. The behavior of the sections in water is shown at the left. Each curve is the mean of two complete series of experiments.

Divalent ions, however, cause marked inhibition of growth. The influence of CaCl_2 on growth in indoleacetic acid (10 mg./l.) is illustrated in Fig. 1; it is evident that the inhibition begins above $10^{-3}\ M$ and varies linearly with the logarithm of the $[\text{Ca}^{++}]$ concentration. A similar effect is exerted by MgCl_2 , as is shown on the same figure; the inhibition is less powerful, beginning only above $3 \times 10^{-3}\ M$, but the shape of the

curve is roughly the same as with calcium. Inhibition of straight growth and curvature by $MgCl_2$ has previously been reported briefly [Table I of Ref. (1)]. Finally, Fig. 1 also shows the growth of the sections in the presence of relatively high concentrations of mannitol; the shape of the curve is again essentially the same.

Because it was felt that exposure to a single divalent salt might bring about undesirable changes due to lack of physiological balance, experiments were carried out with mixtures of $CaCl_2$ and KCl . The inhibition produced by these mixtures is less powerful than with $CaCl_2$ alone.

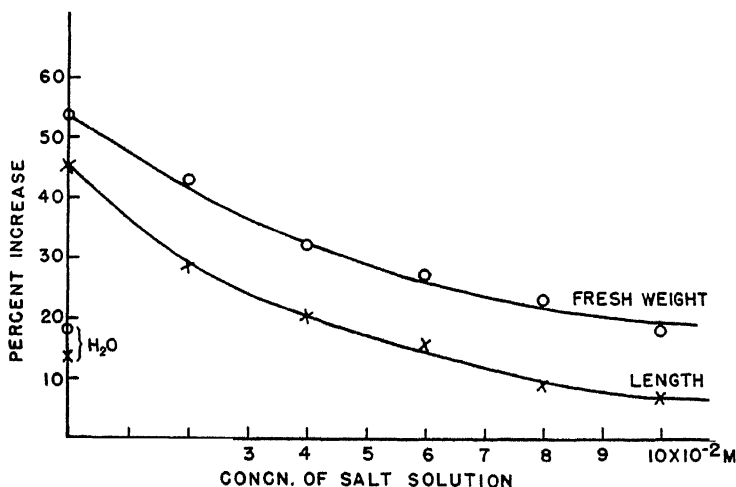


FIG. 2. The elongation and increase in weight of 20-mm. apical sections of etiolated pea stems in indoleacetic acid, 1 mg./l., with the addition of a mixture of 3 parts $CaCl_2$: 1 part KCl . The concentrations shown refer to the total molarity of the mixture. The behavior of the sections in water is shown at the left.

Results with 3 $CaCl_2$: 1 KCl are shown in Fig. 2. The abscissas here are arithmetic, not logarithmic, in order to bring out detail in the critical region. To make the data comparable with those obtained through use of enzyme poisons, these experiments were carried out with indoleacetic acid, 1 mg./l. The point of 50% inhibition is given by a total concentration of almost exactly $4 \times 10^{-2} M$ total salt. With $CaCl_2$ alone (Fig. 1), 50% inhibition occurs at $1 \times 10^{-2} M$. In the salt mixture the sections have a definite tendency to thicken, as is shown by the divergence between the curves for fresh weight and length. However, they remain

TABLE I

Changes in Reducing Sugar During Growth Inhibited by Salt Solution

| Treatment | Growth as per cent of growth in auxin | Reducing sugar | | | |
|-----------------------------------|---------------------------------------|-------------------------------------|-----------------------|------------|---------------------------------------|
| | | Per cent of dry weight ^b | | | Decrease as per cent of initial value |
| | | Range | Number of experiments | Mean | |
| Initial ^c | — | % 16.7–20.4 | 10 | % 18.90 | % — |
| Water | 30 | 11.9–14.3 | 6 | 13.4 | 29.4 |
| Auxin (1 mg./l. IA) | 100 | 11.4–14.1 | 6 | 13.1 | 30.9 |
| Auxin + 4×10^{-2} M salt | 44 | 10.8–13.7 | 7 | 12.8 | 32.5 |
| Auxin + 6×10^{-2} M salt | 37 | 11.0–13.2 | 8 | 12.2 | 35.7 |
| Auxin + 10^{-2} M salt | 15 | 12.8–13.1 | 2 | 12.9 | 31.7 |

^a Salt solution: 3:1 CaCl₂:KCl.^b Dry weight calculated from the fresh weight using the factor 0.067 (1).^c Initial determinations made at time of cutting sections. All others after 24 hr. growth in solution.

in apparently excellent condition. The salt mixture 3 CaCl₂: 1 KCl was used for the experiments below.

Changes in Reducing Sugars

The reducing sugars were extracted from the crushed sections with boiling 80% alcohol, and after treatment with lead acetate and potassium oxalate in the usual way were determined immediately by the Shaffer-Hartman-Somogyi method (5). Standardization of this method against c.p. glucose gave results agreeing closely with those in the literature.

Determinations were carried out on the fresh sections as cut from the seedlings, and after 24 hr. growth either in water, in indoleacetic acid (1 mg./l.) or in indoleacetic acid plus the salt mixture. In all cases the samples used had 15 sections each (20 mm. long). The sections were weighed immediately after cutting, and measured at the end of the growth period. The initial dry weight was calculated from the fresh weight. The inhibitions obtained agreed very well with those shown in Fig. 2; the 4×10^{-2} M salt mixture giving a little more than 50% inhibition.

The collected results are shown in Table I. In the first place it is seen that the content of reducing sugars decreases about equally during growth in water and in auxin, namely about 30% of the initial value. The figure given in the previous paper [Table IV of Ref. (3)] was a decrease of 4.7 from the initial value of 17.5 (percentage of dry weight), i.e., a decrease of 27% during growth. When growth is inhibited by somewhat more than 50% there is very little change in reducing sugars. There is a slight tendency for the value to fall but it is of doubtful significance. In the 0.10 *M* salt, where growth is only 15% of that of the control, the reducing sugar content is virtually identical with that of the control.

These results may be contrasted with those obtained by the use of enzyme poisons (3); for growth inhibition corresponding to that caused by 4×10^{-2} *M* salt the inhibitors would have decreased the reducing sugars by 40–50% (depending on the inhibitor). For inhibition as great as that caused by 0.10 *M* salt the decreases from the initial value would have been 44% (arsenite) to over 65% (fluoride). It is evident, therefore, that no comparable decrease in sugar takes place when growth is inhibited by the salt solutions.

TABLE II
*Changes in Amides of Pea Stem Sections During Growth
in Presence of Nontoxic Inhibitors^a*

| Treatment | Growth as per cent of growth in auxin | Initial ^b dry wt. of sections | Ammonia | | Glutamine | | Asparagine | |
|---|--|--|----------------|--------------|----------------|--------------|----------------|--------------|
| | | | mg./ sample | % dry wt. | mg./ sample | % dry wt. | mg./ sample | % dry wt. |
| Initial ^c | — | 92.2 | 0.115 | 0.13 | 3.10 | 3.36 | 6.20 | 6.75 |
| Auxin (1 mg./l.) | 100 | 76.7 | 0.17 | 0.22 | 1.07 | 1.40 | 8.72 | 11.40 |
| Auxin + salts 6 $\times 10^{-2}$ <i>M</i> ^d | 49.8 | 76.7 | 0.14 | 0.18 | 1.08 | 1.41 | 8.65 | 11.29 |
| Auxin + mannitol 8 $\times 10^{-2}$ <i>M</i> | 40.0 | 77.1 | 0.125 | 0.16 | 1.05 | 1.36 | 8.11 | 11.80 |

^a All data the mean of two determinations on separate samples, each done in duplicate.

^b Calculated from the fresh weight using the factor 0.067.

^c Initial determinations made at the time of cutting sections. All others after 24 hr. growth in solution.

^d Salt solution 3:1 CaCl_2 :KCl.

Changes in Amides

It was shown in the preceding paper (4) that during normal growth of the sections in auxin, about half of the nitrogen of aminoacids is converted into asparagine. This process is inhibited by iodoacetate, arsenite, and fluoride. The free ammonia, glutamine, and asparagine were therefore determined on the sections treated with nontoxic inhibitors. Samples were taken before and after growth in auxin, and after growth in auxin plus mannitol or in auxin plus the CaCl_2 :KCl mixture. The methods were exactly as previously described (4). Two samples of 25 sections each were used in each solution. The growth after 24 hr. at 25°C . was as follows (percentage of initial length):

| | % |
|---|------|
| Indoleacetic acid alone | 54.5 |
| Indoleacetic acid plus mannitol $8 \times 10^{-2} M$ | 27.1 |
| Indoleacetic acid plus 3 CaCl_2 : 1 KCl total $6 \times 10^{-2} M$ | 21.8 |

The results of the determinations are given in Table II. The figures before and after growth in auxin agree well with those given previously. It is clear that neither the consumption of glutamine nor the production of asparagine is significantly changed when growth is inhibited by the mannitol or the salts.

DISCUSSION

These experiments are essentially a group of controls for the ones described in the previous papers. They show that the content of reducing sugars is about the same as in auxin alone when the growth of the stem sections is inhibited without enzyme poisons. They also show that when growth is inhibited without enzyme poisons, the normal increase in asparagine is not interfered with at all. The changes found with iodoacetate, arsenite, and fluoride are therefore due to the action of these inhibitors on enzyme systems and are not secondary results of the growth inhibition *per se*.

The mechanism of growth inhibition by calcium or magnesium is, however, by no means simple. Figure 1 would at first sight suggest that it cannot be simply osmotic, both because of the low concentrations effective and because of the shape of the curve. Simple osmotic inhibition would be expected to vary linearly with concentration. Thus, Fig. 8 of ref. (7) shows a perfectly linear relationship between growth of coleoptile sections and manitol concentration. However, the fact that

mannitol gives essentially the same curve as magnesium indicates that the shape of the curve may be characteristic for the plant material rather than for the inhibitor. That calcium is effective at much lower concentrations than the other two indicates that its action may be more complex. Inhibitory effects of calcium salts on growth have often been described [*e.g.*, Borriß, (2)] but not explained. Effects on permeability are commonly invoked, and the fact that the calcium inhibition is so strongly modified by KCl (*cf.* Figs. 1 and 2) points in the direction of a permeability effect.

SUMMARY

The growth of isolated pea stem sections in auxin solution is strongly inhibited by calcium ions, and somewhat less so by magnesium ions or by mannitol. The inhibition varies almost linearly with the logarithm of the inhibitor concentration.

A mixture of CaCl_2 and KCl gives less powerful inhibition than CaCl_2 alone, and this varies more nearly linearly with the concentration.

Using the latter mixture, the changes in reducing sugar during growth and inhibition were studied.

Growth can be inhibited up to 85% by the salt mixture with only slight decreases in reducing sugar below the values in auxin controls.

Similarly, growth can be inhibited at least 50% with no change in the asparagine content from that in auxin controls. Inhibition by mannitol also had no effect on the asparagine formation. In all cases, the asparagine content was increased about 65% and the glutamine content decreased about 50% during the growth period.

The decreases in reducing sugar and asparagine which follow treatment with arsenite, iodoacetate, or fluoride are therefore due to the effects of these inhibitors on enzyme systems, and are not secondary results of the growth inhibition.

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LETTERS TO THE EDITORS

The Formation of Pyrophosphate from Adenosine Triphosphate in the Presence of a Snake Venom

In previous papers (1, 2) it was shown that all snake and arthropod venoms hitherto investigated are able to dephosphorylate adenosine triphosphate (ATP) in the presence of magnesium ions. Under most conditions the reaction velocity drops considerably or completely after the release of one molecule of phosphoric acid.

Gulland and Jackson (3) discovered the presence of a 5-nucleotidase in four snake venoms. In extending the studies of these authors, it could be demonstrated (4) that so far all snake venoms contain such an enzyme, including one or more species from *Bungarus*, *Denisonia*, *Naja*, *Notechis*, *Pseudechis*, *Sepedon* (colubrids), *Aghkistrodon*, *Bitis*, *Bothrops*, *Crotalus*, *Sistrurus*, *Trimeresurus*, and *Vipera* (viperids). The variations in activity of this enzyme in different venoms, its activation by cobaltous ions, and the fact that it is not inhibited by α , α' -dipyridyl clearly indicate that it is distinct from the ATP-splitting enzyme mentioned above.

The question arose as to whether (a) the phosphoric acid liberated from ATP in the presence of venoms is split off from the end of the molecule with formation of adenosine diphosphate (ADP), or whether (b) pyrophosphate and adenylic acid are produced, the latter then being dephosphorylated by the 5-nucleotidase. There are many results which can hardly be interpreted otherwise than by the assumption of mechanism (a). But, under certain conditions, e.g., low substrate concentration, mechanism (b) seems to take place.

In order to test possibility (b), the venom of *Bitis gabonica*, by far the most active source of the ATP-splitting enzyme (2), was used. In the presence of 4 millimolar Mg^{++} and glycine buffer of pH 8.3 $Q_{\mu P}$ -values (μ moles phosphoric acid/mg. venom/hr.) above 200 were recorded (2).

When 14.8 μ moles of ATP was incubated for 0.5 hr. with 1 mg. of this venom under the conditions already mentioned, 14.2 μ moles of inorganic phosphate (1.0 molecule) was liberated (all blanks having been subtracted, which never exceeded 3%), leaving 26.0 μ moles of acid-labile phosphoric acid (1.8 molecules). After a short heating of the solution, its volume of 30 ml. was reduced *in vacuo* to 2 ml. and this was deproteinized with an equal volume of 10% trichloroacetic acid. The supernatant fluid was treated with cadmium acetate. The ensuing precipitate was washed once on the centrifuge with distilled water. In the resuspended precipitate only traces of free phosphate were detected (1% of the total phosphate), while after the usual 7-min. acid hydrolysis 26.8 μ moles (1.8 molecules) appeared. The hydrolysate was checked spectroscopically, and no specific absorption between 250 and 280 $m\mu$ could be detected, indicating, that no ATP or other adenine compounds were in the cadmium precipitate.

Thus, under certain conditions, the venom of *Bitis gabonica* is able to split ATP into adenylic acid and pyrophosphate. Adenylic acid subsequently is dephosphorylated by a 5-nucleotidase which usually is the most active enzyme in the complement of phosphatases of a given venom.

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The Relation Between the Proteolytic and Blood Clotting Activity of Snake Venoms

The relation between the proteolytic activity and blood clotting effect of certain snake venoms has already been demonstrated (1). We reinvestigated this problem by more accurate methods, using venoms all of which possess a strong clotting effect both on plasma and purified fibrinogen. The venoms used were samples obtained from many specimens. We also investigated a product obtained from *Bothrops atrox* venom by precipitation with acetone and heating. Table I presents some of our results.

TABLE I

The Proteolytic and Blood Clotting Activity of Different Snake Venoms

| Venom ^a | Proteolytic activity ^b | Coagulating activity ^c | Ca/Pa ^d |
|----------------------------------|-----------------------------------|-----------------------------------|--------------------|
| <i>Lachesis muta</i> | 14.0 | 6.0 | 0.43 |
| <i>Bothrops jararacussu</i> | 6.6 | 1.5 | 0.23 |
| <i>Bothrops atrox</i> | 5.8 | 3.5 | 0.60 |
| <i>Bothrops neuwiedii</i> | 5.1 | 1.7 | 0.33 |
| <i>Bothrops jararacussu</i> | 3.9 | 2.6 | 0.67 |
| <i>Bothrops alternata</i> | 1.5 | 2.4 | 1.60 |
| <i>Bothrops cotiara</i> | 1.2 | 1.5 | 1.25 |
| <i>Crotalus terr. terrificus</i> | 1.0 | 1.0 | 1.0 |

^a For both proteolytic and clotting activity measurements 1 mg./ml. saline solutions were used. The solutions used in clotting tests also contained 0.5% phenol.

^b The proteolytic activity determinations were made by the method of Anson (2) on hemoglobin substrate and the results are expressed in mequiv. of tyrosine $\times 10^4$.

^c The coagulating activity is expressed in reciprocal minutes of the clotting time. The measurements were carried out with oxalated rabbit plasma.

^d Ca/Pa represents the ratio: coagulating activity/proteolytic activity.

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In this table the venoms have been listed according to decreasing proteolytic activity. With two exceptions this gives also a decreasing coagulating effect. The ratio: coagulating activity/proteolytic activity is characteristic for each type of venom. This ratio might therefore be useful for the differentiation of venoms of unknown or uncertain origin. The exceptions mentioned above are the venoms of *Bothrops jararacussu* and *Bothrops neuwiedii*. These venoms show a very low coagulating power as compared with the proteolytic activity. This is expressed in a low figure for the ratio between these activities.

On the other hand some of the venoms with low proteolytic activity show a disproportionately smaller decrease in the coagulating activity resulting in a rather high figure for the ratio. This applies especially to the venoms of *Bothrops alternata* and *Bothrops cotiara*.

It seems very probable that the proteolytic activity is due to the same substance as is responsible for the coagulating activity, as experiments in which the activities of natural and of precipitated and heated venom of *B. atrox* were compared showed that the proteolytic activity and the coagulating activity are decreased to the same extent. The mechanism of the coagulating action of these different venoms is not known, but it was found recently (3) that there is a difference between the direct action of thrombin on fibrinogen and that of snake venoms.

Our experiments show a close relation between the proteolytic and blood clotting activity of snake venoms.

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Effect of Vitamin B₁₂ Supplementation on the Pig

Novak and Hauge (1) worked on a new growth factor for the rat which they tentatively called vitamin B₁₂. In the study reported herein a B₁₂ concentrate, prepared according to the method of Austin and Boruff (2), was studied with the young growing pig.

A control ration of ground yellow corn 57%, peanut meal 41.5%, bone meal 0.5%, limestone 0.5%, and salt-trace mineral mixture 0.53% (salt-trace mineral mixture consisted of iodized salt 50 lb., manganese sulfate 921 g., ferrous sulfate 398 g., copper sulfate 125 g., and cobalt carbonate 10 g.) was used. Vitamins A and D and seven B-complex vitamins, which the pig has been shown to need in the ration, were added at levels previously described by Cunha *et al.* (3). In Expt. 1, four purebred Duroc pigs were fed in each lot. Five grade native pigs (of mixed breeding) were fed

in each lot in Expt. 2. The pigs were fed on concrete floors which were washed once daily.

In Expt. 1, no growth response was obtained by addition of the B₁₃ concentrate during the first 16 days of the trial, but during the following 46 days the pigs fed B₁₃ gained 25% faster than the controls. The average daily gains for the entire 62-day trial show that the pigs fed the B₁₃ concentrate gained approximately 19% faster than those on the basal ration. See Table I.

TABLE I
Results in Experiment 1

| Lot no. | Av. starting weight | Ration fed | Av. daily gain first 16 days | Av. daily gain next 46 days | Av. daily gain for 62 days |
|---------|---------------------|---|------------------------------|-----------------------------|----------------------------|
| | <i>lb.</i> | | <i>lb.</i> | <i>lb.</i> | <i>lb.</i> |
| 1 | 36.5 | Basal+B ₁₃ ^a | 1.09 | 1.00 | 1.02 |
| 2 | 37.7 | Basal+B ₁₂ +B ₁₃ ^b | 1.08 | 1.25 | 1.21 |

^a Vitamin B₁₂ concentrate (Fuller's earth) from Dr. D. F. Green, Merck & Co., Rahway, New Jersey (contained 12.5 mg. of B₁₂/lb.). Fed at a level of 45.4 g./100 lb. of feed.

^b Vitamin B₁₃ liquid concentrate fed at a level of 3 ml./100 lb. of feed. Obtained from Dr. R. A. Rasmussen, Hiram Walker & Sons, Inc., Peoria, Illinois.

The results obtained in Expt. 2 showed that for the first 45 days on experiment there was no benefit from B₁₃ supplementation. However, after that period of time, the pigs fed B₁₃ gained approximately twice as rapidly as the controls. The average daily gains for the 70-day period showed that B₁₃ supplementation increased the rate of gain. Evidently, the level of B₁₃ supplementation in lot 4 was high enough since the pigs fed twice as much of the B₁₃ concentrate (lot 5) did not gain any faster than the pigs in lot 4. See Table II.

TABLE II
Results in Experiment 2

| Lot no. | Av. starting weight | Ration fed | Av. daily gain first 15 days | Av. daily gain next 25 days | Av. daily gain for 70 days |
|---------|---------------------|--|------------------------------|-----------------------------|----------------------------|
| | <i>lb.</i> | | <i>lb.</i> | <i>lb.</i> | <i>lb.</i> |
| 3 | 38.9 | Basal+methionine ^a +B ₁₂ ^b | 0.90 | 0.56 | 0.78 |
| 4 | 40.5 | Basal+methionine+B ₁₂ +B ₁₃ ^c | 0.90 | 1.14 | 0.98 |
| 5 | 40.1 | Basal+methionine+B ₁₂ +B ₁₃ | 0.86 | 1.05 | 0.93 |

^a DL-Methionine fed at 0.15% of the ration. Supplied through the courtesy of Dr. H. J. Prebluda, U. S. Industrial Chemicals, Inc., New York, N. Y.

^b B₁₂ fed in all lots at same level as in trial 1 (Table I).

^c B₁₃ liquid concentrate fed at a level of 3 ml./100 lb. feed in lot 4 and at twice that level in lot 5.

It is difficult to explain the delay necessary before the B_{13} concentrate benefited growth. In Expt. 2 it took 45 days before B_{13} supplementation began to increase the rate of gain. A possible explanation for the delayed response of B_{13} supplementation on rate of gain is that a certain amount of time may be required to deplete pigs to the point where supplementation will be of benefit. However, once the pigs on the basal ration become depleted they fail to gain as rapidly as the pigs fed B_{13} . Although the pigs in lot 5 were fed twice as much B_{13} as those in lot 4, the growth response above that of the basal lot occurred in both lots 4 and 5 at the same time. This indicates that the greater rate of gain was not due to an increased accumulation of B_{13} in the body, but was probably associated with the depletion and consequent reduction of gain for the pigs on the basal ration.

These data show that a B_{13} concentrate was of benefit in growth for the pig. However, variation existed in the length of time required before B_{13} supplementation began to increase the rate of gain, perhaps indicating that B_{13} is stored by the pig.

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Maternal Sulfate Utilized by Mammalian Embryos and Suckling Young¹

It has been shown that sulfate ion is bound *in vitro* by embryonic tissues (5), certain adult human tissues (3), and by granulation tissue from healing muscle wounds (4). In a subsequent paper (6) it will be shown that labeled sulfate is fixed in connective tissue and in granulation tissue *in vivo*. Further work as yet unpublished would indicate that sulfate sulfur is probably fixed as chondroitin sulfate in the connective tissue ground substance. It has been suggested by Dziewiatkowski *et al.* (1) that injected sulfate sulfur may be used by the suckling rat in the synthesis of chondroitin sulfate of knee-joint cartilage. A preliminary report by Hanahan *et al.* (2) indicated that labeled sulfate from the maternal rat was not found in the fetal tissues.

Since sulfate appeared to be utilized for connective tissue formation *in vitro* and *in vivo* by embryonic and adult chickens, it was considered desirable to determine whether sulfate of the maternal organism is utilized by mammalian embryos and suckling young.

¹ This investigation was supported in part by a grant from the American Cancer Society on recommendation by the Committee on Growth of The National Research Council, and in part by the Nutrition Foundation, Inc.

TABLE I

Utilization of Sulfate by Rat Embryos in Utero

In vivo fixation of radioactive sulfate in the tissues of the embryonic rat; pregnant female injected with 10^7 counts of carrier-free sulfate, $\text{Na}_2\text{S}^{35}\text{O}_4$, intramuscularly into thigh 7 days previous to removal of embryos. Tissues were washed prior to assay (6).

| Embryonic Tissue | $\text{Na}_2\text{S}^{35}\text{O}_4$ in tissue (counts/100 mg. wet tissue) |
|------------------|---|
| Heart | 80 |
| Abdominal aorta | 1000 |
| Kidney | 85 |
| Skeletal muscle | 290 |
| Femur (entire) | 2700 |

Utilization of Sulfate from Milk

In vivo sulfate fixation in the tissues of suckling rats. The mother was injected intramuscularly with 10^7 counts of $\text{Na}_2\text{S}^{35}\text{O}_4$ on day following birth of young. The young were sacrificed 1 week later, and the tissues assayed for sulfate.

| Tissue | $\text{Na}_2\text{S}^{35}\text{O}_4$ in tissue | |
|------------------|--|--|
| | Unwashed tissue counts/100 mg. wet tissue | Washed tissue counts/100 mg. wet tissue |
| Heart ventricle | 45 | 17 |
| Thoracic aorta | 90 | 35 |
| Kidney | 50 | 40 |
| Shaft of tibia | 300 | 280 |
| Condyle of tibia | 3500 | 3500 |

In a preliminary experiment, carrier-free $\text{Na}_2\text{S}^{35}\text{O}_4$ was injected into the thigh muscles of pregnant and nursing rats. Seven days later, tissues of the embryos and suckled young were assayed for radioactive sulfate.² The data are presented in Tables I and II, respectively. The method employed has been described elsewhere (5, 6).

SUMMARY

The results indicate: (a) that sulfate is transferred across the placental barrier and is utilized in tissue formation by the embryo, and (b) that sulfate secreted in the milk, is utilized for tissue synthesis by the suckling young.

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The Replacement by D-Lysine of *p*-Aminobenzoic Acid as Growth Factor for *Lactobacillus arabinosus* 17-5

The results to be presented here show that D-lysine can replace *p*-aminobenzoic acid (PABA) as a growth factor for *Lactobacillus arabinosus* 17-5. The growth response of this organism to PABA has been reported previously (1, 2, 3).

The materials and methods used are those described previously (3) with the addition, when desired, of lysine samples to casein hydrolysate medium to replace PABA. The following lysine stock solutions in distilled water were prepared for these

TABLE I

Growth Turbidity Readings with Klett-Summerson Colorimeter using Filter No. 54

| Systems, hr. | | 20 | 48 | 64 | 88 |
|----------------------------|---------------|----|-----|-----|-----|
| | conc./ml. | | | | |
| Control | — | 0 | 0 | 10 | 49 |
| PABA | 0.01 μ g. | 87 | 214 | 218 | 240 |
| DL-Lysine | 4.8 mg. | 0 | 119 | 149 | 198 |
| D-Lysine | 2.4 mg. | 30 | 120 | 150 | 202 |
| L-Lysine | 2.4 mg. | 0 | 10 | 25 | 100 |
| D-Lysine plus | 2.4 mg. | 40 | 130 | 180 | 214 |
| L-Lysine | 2.4 mg. | | | | |
| PABA plus | 0.01 μ g. | 30 | 182 | 191 | 220 |
| sulfanilamide | 1.0 μ g. | | | | |
| D-Lysine ^a plus | 2.4 mg. | 0 | 0 | 0 | 0 |
| sulfanilamide | 1.0 μ g. | | | | |

^a Also D- + L- and DL-lysine stimulations are similarly susceptible to sulfanilamide.

additions: (1) DL-Lysine monohydrochloride, (2) L-lysine monohydrochloride, and (3) two samples of D-lysine dihydrochloride, one commercially obtained,¹ the other sample a gift from Dr. J. P. Greenstein (5).²

¹ The commercially obtained D-lysine had been resolved from the racemic mixture chemically in a method such as that described by Berg (4).

² The authors are indebted to Dr. J. P. Greenstein of the National Cancer Institute, United States Public Health Service, Bethesda, Md., for the invaluable supply of the D-amino acids used in these experiments.

From Table I it can be seen that DL-lysine will replace the PABA requirement of this organism and that this activity would appear to reside in the D-lysine isomer of this racemate. The D-lysine would appear to be more active than the racemate during the early stages of growth. Also it is of interest to note that this stimulation by D-lysine, which is structurally dissimilar, is highly susceptible to sulfonamide inhibition.

Both samples of D-lysine gave duplicate results although resolved by different methods, and several other D-amino acids resolved enzymatically, also kindly supplied by Dr. Greenstein, did not show this activity. That the stimulation of growth by D-lysine in the absence of PABA is not due to a contamination by PABA is evident from the following observations: (a) The D-lysines prepared by two distinct methods stimulate growth to an equal degree, and stimulation by synthetic DL-lysine is directly related to its D-lysine content by weight. It is possible but it seems highly unlikely that these various samples of D-lysine and DL-lysine are contaminated by PABA to an equal degree; and (b) the growth stimulation by PABA added to L-lysine was completely eliminated by charcoaling followed by two recrystallizations; this identical treatment of D-lysine failed to affect to any degree the growth-stimulating activity.

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Studies on the Metabolism of L-Valine in Phlorizinized and Fasted Animals

Evidence for the glyconeogenic property of valine is based principally on two sets of experiments: first, oral administration of DL-valine to fasting rats gave rise to a small but significant increase in liver glycogen (1), and second, in phlorizinized dogs, administration of either D- or L-valine led to an increase in urinary glucose corresponding to the utilization of three of the five carbon atoms (2).

This report presents data from two experiments designed to study these conversions by means of L-valine labeled with C¹³ in the methyl groups. Two phlorizinized rats (3) and two fasted 48 hr., each received by stomach tube 73.6 mg. of L-valine (C¹³ atom-% excess, 9.95) hourly for a total of 8 hours. Glucose was isolated and determined by known methods (3, 4).

Data on the phlorizinized rats are presented in Table I. Liver and muscle glycogen were isolated (5) from the fasted rats and showed an average C¹³ atom-% excess of

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TABLE I
Administration of Methyl-Labeled L-Valine to Phlorizinized Animals

| | Animal 1 | | | | | Animal 2 | | | | |
|--|------------------|------|-------------------|-------------------|------|----------|------|-------------------|-------------------|------|
| | Day of phlorizin | | | | | | | | | |
| | 1 | 2 | 3a ^{a,c} | 3b ^{a,d} | 4 | 1 | 2 | 3a ^{a,c} | 3b ^{a,d} | 4 |
| Urinary glucose, mg. | 725 | 836 | 194 | 518 | 637 | 768 | 672 | 222 | 562 | 687 |
| Urinary nitrogen, mg. | 232 | 264 | 78 | 190 | 245 | 256 | 212 | 81 | 180 | 255 |
| Glucose/nitrogen ratio | 3.12 | 3.16 | 3.00 ^b | | 2.60 | 3.00 | 3.17 | 4.10 ^b | | 2.69 |
| “Extra sugar,” mg. | 127 | | | | | 220 | | | | |
| Conversion of valine to “extra” glucose, % | 28 | | | | | 48 | | | | |
| C ¹³ , atom-% excess: | | | | | | | | | | |
| Administered valine | 9.95 | | | | | 9.95 | | | | |
| Isolated glucose (8 hr. urine) | 0.874 | | | | | 0.972 | | | | |
| Isolated glucose (8-24 hr. urine) | 1.054 | | | | | 1.076 | | | | |
| Isolated glucose (24-48 hr. urine) | 0.090 | | | | | 0.088 | | | | |
| Recovery of isotope in urinary glucose, % | 10.3 | | | | | 11.7 | | | | |
| Recovery of isotope in expired CO ₂ , % | 10.6 | | | | | 12.1 | | | | |

^a L-Valine administered to each animal was 589 mg.

^b Corrected for exogenous nitrogen.

^c Urine collected first 8 hr.

^d Urine collected 8th to 24th hr.

0.54 and 0.12, respectively, which corresponded to 0.85 and 0.10% recovery of the administered isotope. C¹³-recovery from carbon dioxide expired during the first 8 hours was 12.2%. The data presented show that the methyl group of L-valine can enter into glucose of glycogen formation under the conditions of these experiments and that in part is converted to carbon dioxide. The isolated glycogen and glucose are being degraded to determine the position of the isotopic carbon in these substances.

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A Derivative of Cozymase as Activator of Fermentation¹

Besides the known cofactors of fermentation several other activators play a role in regulating the speed of fermentation of yeast cells in various phases [see, e.g., (1,2)]. We have found a new factor which is formed by treatment of cozymase with very dilute carbonate solution at pH 10.4 at room temperature for several minutes. At pH 9.9 or 10.9 hardly any effect is obtained.

Activation of fermentation occurs only in the presence of phosphate and with an excess of adenosinetriphosphatase in preparations of yeast cells, either dried as recently described (3), or frozen in liquid air (4). If such a yeast is washed out with a phosphate solution, which removes the larger part of the preformed coenzymes, and is taken up in a dilute suspension (10–20 mg. dry weight/1 ml.) in a solution of glucose and phosphate, fermentation is very low without added cofactors (dried yeast), or zero (frozen yeast). With cofactors including cozymase in optimal amounts (about 5×10^{-4} M), this fermentation is tripled. If carbonate-treated cozymase is used instead, a fermentation is obtained which is three to six times higher than with untreated cozymase. The actual increase depends on the amount of cozymase taken and also on the concentration of the yeast, because in the absence of activators the fermentation rate does not rise proportionally to the density of the yeast cells but nearly to the second power; this is probably because of the increased concentration of the dissolved intermediaries of fermentation and other activating substances. This stimulating effect of the carbonate-treated cozymase becomes correspondingly smaller in more highly concentrated yeast suspensions.

A similar activation is obtained with relatively large amounts (1×10^{-3} M) of nicotinamide mononucleotide² (NMN) (5,6). This NMN may therefore be one component of the activating system, but it cannot be the only one because mere traces of NMN would form at pH 10.4. Nicotinamide is in itself ineffective. On the other hand, boiled yeast juice gives under the same conditions a similar and more lasting increase in fermentation. However, boiled yeast juice is too complex a system for elucidating the reasons of activation.

A typical experiment with dried yeast is reproduced in Fig. 1. A strong activation by carbonate-treated cozymase is always obtained with various preparations of yeast, brewer's yeast, yeast K (7)³, and various preparations of cozymase, 83% pure from Sigma Chemical Co., or 55% pure from Schwarz Laboratories. With unwashed yeast the activation likewise occurs but is smaller. No activation occurs in yeast extracts.

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² We thank Dr. A. Kornberg for the sample of NMN.

³ We thank Dr. S. Spiegelman, Department of Bacteriology, University of Illinois, Urbana, for an agar culture of yeast K, and Dr. M. Sevag, Department of Bacteriology, University of Pennsylvania School of Medicine, Philadelphia, for subculturing this yeast.

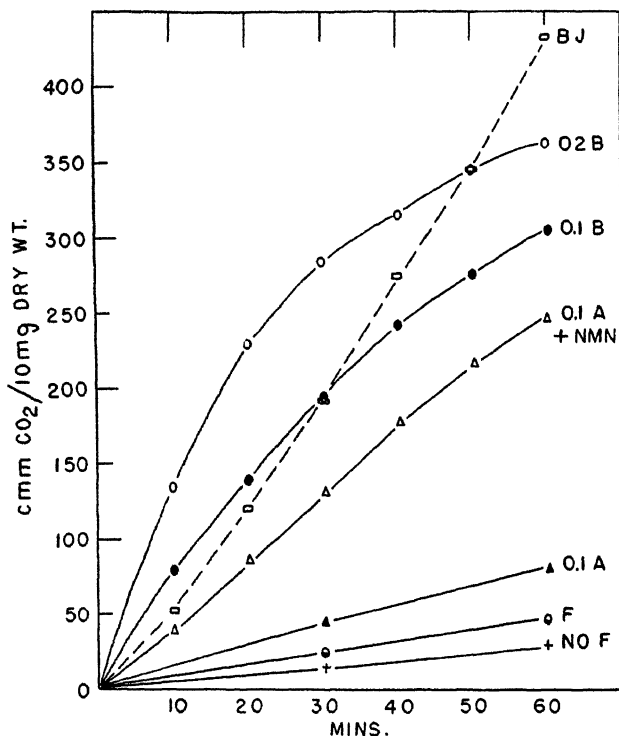


FIG. 1. Fermentation in washed dried brewer's yeast (Expt. P-142). Yeast washed with 50 ml. 0.05 *M* phosphate. Total volume in each vessel: 1.6 ml. with 20 mg. dried yeast. In the main compartment of each vessel there are 8×10^{-3} *M* phosphate, 5×10^{-3} *M* MgSO₄ and either 0.3 ml. water or 0.3 ml. boiled yeast juice. Tipped in at time 0 min., 8 mg. glucose + water or cofactors to 0.6 ml. Mixture of cofactors contains adenosine triphosphate with 30 μ g. 7 min. P, adenosine diphosphate with 15 μ g. P, acetaldehyde 0.3 mg. and various additions of cozymase as given in the single curves.

+, no *F*: no cofactors added; \odot , *F*: cofactors without diphosphopyridine nucleotide (DPN) added; \blacktriangle , 0.1 *A*: untreated DPN, 8×10^{-1} μ mol.; \triangle 0.1 *A* + NMN: the same + 1.4 μ mol. NMN; \bullet , 0.1 *B*: 8×10^{-1} μ mol. pretreated DPN; \circ , 0.2 *B*: 1.6 μ mol. pretreated DPN; \square *B. J.*: boiled yeast juice prepared from the same dried yeast (1:5) and added in the main compartment.

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Breakdown of Infecting Coliphage by the Host Cell¹

The present letter is a report of work carried out with T_2r+ bacteriophage, labeled with P^{32} , active on *Escherichia coli* B, to determine the fate of the infecting particle. The virus was labeled and purified in a manner similar to that described by Putnam and Kozloff (1) for T_6 bacteriophage, the P^{32} being contained almost entirely in the nucleic acid of the virus.

Cultures of *E. coli* grown in Difco Bacto-Tryptose broth to 2×10^8 cells/ml. were infected with the labeled bacteriophage and aerated at 37°C . At intervals measured amounts of the culture were withdrawn and concentrated trichloroacetic acid (TCA) was added to make a final concentration of 5%. The mixture was centrifuged and the amount of P^{32} in the supernatant was used as a criterion of the extent of breakdown of nucleic acid in the infecting virus particle. The amount of P^{32} present in the TCA extract was calculated as a percentage of the P^{32} added in the infecting bacteriophage. Figure 1 illustrates average curves relating this quantity to time after addition of radioactive bacteriophage to the cells for different experimental conditions.

Curve I shown the release of P^{32} when cells were singly infected with radioactive bacteriophage, 1 virus particle to 5 cells, and curve II when 2 to 10 virus particles/cell were added to the culture. When cells were infected with purified but not radioactive T_2r+ bacteriophage, and labeled bacteriophage was added at some later time, the breakdown of labeled bacteriophage was as shown in curve III. Curve III was reproduced whether the initial infection was 2 to 10 virus particles/cell or the labeled bacteriophage was added at ratios of 0.2 to 60 particles/cell between 2-120 min. following initial infection.

The following interpretation is proposed tentatively. Infection of the cells stimulates some mechanism, at present unknown, so that other virus particles becoming attached to the cell after a short interval are each broken down to the extent of about 52% as shown in curve III. The toe of curve I represents the conditions when the cell is singly infected, namely, about 4% breakdown of the infecting particle. Multiple infection as shown by curve II represents an intermediate state; the secondary rise of curve I might then be explained as a multiple infection of the type of curve II resulting from the release of first generation of virus and adsorption on uninfected cells.

When cells were multiply infected with radioactive bacteriophage about 20% of the P^{32} appeared in the progeny. However, when the labeled virus was added 15

¹ This investigation was aided by a grant from the National Cancer Institute of Canada.

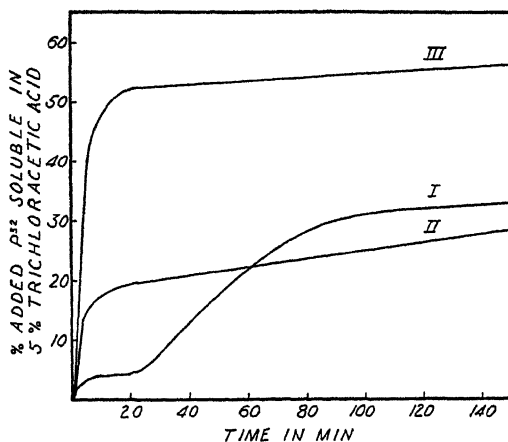


FIG. 1. Breakdown of infecting coliphage by the host cell.

minutes after multiple infection with nonradioactive bacteriophage the progeny contained only 1% of the added radioactivity. Apparently, virus adsorbed after infection is initiated contributes little to the formation of progeny.

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A Note on a New Antibiotic

A strain of *Streptomyces*, designated NA232-M1 in our culture collection, has been isolated from a soil sample collected at North Chicago, Illinois. This organism when grown in shaken culture or submerged tank culture on a medium containing soybean oil meal, cerelese, sodium chloride, and calcium carbonate, produces an antibiotic which is extremely active against acid-fast bacteria, gram-negative bacteria, and a few gram-positive bacteria. The antibiotic is produced in yields of several hundred milligrams per liter of fermentation culture. Paper-strip chromatograms on samples of clarified beer indicated an antibiotic at a position different from that of streptomycin, mannosidostreptomycin, dihydrostreptomycin, neomycin, or streptothricin. A purified preparation of the antibiotic assaying 600 units/mg. by a streptomycin assay procedure against streptomycin sulfate as a standard inhibits the growth of *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, *Aerobacter aerogenes*, *Eberthella typhosa*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Neisseria catarrhalis*, *Brucella abortus*, *Mycobacterium tuberculosis* 607, *Mycobacterium phlei*, and *Corynebacterium diphtheriae*, in concentrations of from 0.5 to 5 $\mu\text{g.}/\text{ml.}$ in laboratory media. The presence of 10% beef

serum in the medium has no effect on the antimicrobial activity. The hydrogenated antibiotic has the same antimicrobial spectrum as the original material.

To purify the antibiotic, the beer was acidified to pH 2 with sulfuric acid, filtered, neutralized, and the antibiotic adsorbed on a column of Amberlite IRC-50. The resin was eluted with 0.5 *N* H₂SO₄ and a crude preparation obtained from the neutralized eluate by evaporation and precipitation with acetone. The crude product was further purified by chromatography on Darco G-60-Celite 545. Preparations of about 700 streptomycin units/mg. and $[\alpha]_D = -79^\circ$ have been obtained.

The antibiotic resembles streptomycin (1) chemically but can be differentiated from streptomycin, mannosidostreptomycin, dihydrostreptomycin, streptothricin, and neomycin by paper chromatography. In common with streptomycin it gives a positive Sakaguchi test, a positive test for *N*-methylglucosamine, and liberates methylamine on alkaline hydrolysis and streptidine on acid hydrolysis. In contrast to streptomycin, the antibiotic contains no C'-methyl group (Kuhn-Roth determination),¹ and upon alkaline hydrolysis, in place of maltol, a new compound is formed which appears to be an isomer of kojic acid. This compound melts at 154–157°C. It gives a positive FeCl₃ test and shows strong absorption in the ultraviolet. $E_{1\text{ cm}}^{1\%}$ 696 at 274 mμ in 0.01 *N* HCl and $E_{1\text{ cm}}^{1\%} = 597$ at 319 mμ in 0.05 *N* NaOH.

Anal. Calcd. for C₆H₈O₄: C, 50.71; H, 4.26.

Found: C, 50.87; H, 4.21.

The antibiotic can be hydrogenated to form a new antibiotic which has been obtained as a crystalline base.

Anal. Calcd. for C₂₁H₄₁N₇O₁₃·H₂O: C, 40.84; H, 7.02; N, 15.88.

Found: C, 40.74; H, 6.88; N, 15.94.

From the above data it may be postulated that the antibiotic differs from streptomycin only by one oxygen atom which is present as a hydroxyl group on the methyl group of the streptose portion of the molecule.

The toxicity of a preparation of antibiotic NA232-MI assaying 675 units/mg. was determined in comparison with a commercial lot of streptomycin sulfate. By intravenous injection in mice the approximate LD₅₀ of this new antibiotic was found to be 104,000 units/kg. as compared with 100,000 units/kg. for streptomycin. By subcutaneous injection the corresponding values were 640,000 units/kg. and 500,000 units/kg., respectively. Thus the toxicity of NA232-MI appears to be equal to that of streptomycin by intravenous injection and somewhat lower by the subcutaneous route. The symptoms in the toxic doses were ataxia and sleep-like depression and were the same for both antibiotics; no deaths occurred later than a few hours after the injection.

Ten mice were given $\frac{1}{2}$ of the LD₅₀ of streptomycin or NA232-MI, respectively, intravenously for 9 days. After this $\frac{1}{2}$ of the LD₅₀ dose was administered for 6 days and then $\frac{1}{4}$ of the LD₅₀ for an additional 3 days. During the last period 4 out of the 10 streptomycin-treated mice died but none of those treated with NA232-MI. Further studies to determine the relative toxicity of the antibiotic, and especially its possible effect upon the 8th nerve, are under way.

¹ Analyses by E. F. Shelberg and associates.

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Book Reviews

The Chemistry of Organic Medicinal Products. By GLENN L. JENKINS, Professor of Pharmaceutical Chemistry and Dean of the School of Pharmacy, Purdue University, and WALTER H. HARTUNG, Professor of Pharmaceutical Chemistry, School of Pharmacy, The University of North Carolina. 3rd Ed., John Wiley and Sons, Inc., New York, N. Y., 1949. ix + 745 pp. Price \$7.50.

In the preface to the third edition of "The Chemistry of Organic Medicinal Products" Drs. Jenkins and Hartung state that they have adhered to the original objectives of the first edition of organizing the large number of organic medicinal compounds according to the accepted scheme of chemical classification and of dealing with the methods of preparation, properties, and descriptions of the more important compounds of each class. Entirely new or completely rewritten sections on such subjects as the antibiotics, antimalarials, sulfonamides, amino acids, antihistaminic drugs, and enzymes have been added. Every chapter has been thoroughly revised. "Although it presents many elementary aspects of the subject so that the beginner in this field of study can readily find his way to an understanding of the more advanced topics, it is intended primarily for students in the more advanced courses in pharmaceutical, chemical, biological, and medicinal science."

Unfortunately the high aims set forth in the preface seem to fall short of accomplishment. In the novice, much misinformation on organic chemistry will be implanted and the treatment of the chemistry involved is entirely too sketchy and cursory to awaken curiosity on the part of the more mature investigator. Particularly to be regretted is the confusion attendant upon the effort to utilize the newer theories and terminology of organic chemistry. For example the concept of isosterism has been introduced with most unfortunate connotations. The statement that "thiophene is more isosteric with benzene than with furan" is certainly meaningless and can only increase the chaos that has resulted from indiscriminate overuse of the isoster concept. In too many places the double ended single arrow, commonly employed to denote resonance, has been used for expressing tautomerism. The principles governing orientation in the benzene series are based on the old Crum Brown-Gibson rule.

Perhaps more serious is the number of misstatements of chemical fact. For example the statement that amination of a halide is not applicable for the introduction of an amino group into an aromatic nucleus is surprising in view of the success attending the Dow process for the manufacture of aniline. The process given for the synthesis of paludrine is not the one commonly used in commercial production. The impression that catalytic reduction of nitrogen functions such as oximes or nitriles is unsatisfactory for the synthesis of primary amines fails to take into account the success which has accompanied such reductions in the presence of an excess of ammonia. The atebtrin synthesis as given is not in accord with the facts.

In several places deviations from historical accuracy were noted. The actual discovery of chloroform by Samuel Guthrie in this country which preceded the work of

Liebig and Soubeiran is not mentioned. Berzelius, to whom the recognition of enzymes is credited in 1885, actually died in 1848.

The new material which appears in this edition has been selected from topics of current interest. However, several errors were noted in the section on enzymes. A general discussion of this complex subject presents a problem in a book of this size. It does seem that in the interests of condensation, misleading statements have crept in accompanied by much incorrect information.

In the section on the sulfonamides, one wishes that more emphasis had been given to the relationship between the physicochemical properties of the drugs and their action. It was also rather surprising to find several true sulfones appearing in the sulfonamide section rather than in the chapter on sulfones in which are found such non-sulfones as BAL and thioglycerol.

Throughout the book, apparently in a worthy effort at condensation, too many sweeping generalizations are found. It would be more desirable to utilize some of the space devoted to a detailed enumeration of physical properties of the host of drugs listed for a more critical presentation of facts.

For a third edition, the number of typographical errors which have crept through is appalling. Many formulas are in error and faulty grammatical constructions are frequent.

Perhaps the most valuable feature of the book is the inclusion of an added number of direct references to original articles and a useful list of pertinent book, review, and journal citations at the end of each chapter. With the aid of these, the reader interested in pursuing a given field further should have no difficulty in finding adequate reading matter for this purpose. It is rather surprising to find DeKruif's "Male Hormone" listed as a reference work in a book dealing with chemistry.

The book will also be of value to those who find themselves confused by the camouflage of trade names under which most drugs are marketed and which in general serve admirably to conceal their chemical nature. The index is comprehensive and one can easily run down the chemical nature of a drug with its aid.

ROBERT C. ELDERFIELD, New York, N. Y.

Biochemical Society Symposia No. 3; Partition Chromatography. Edited by R. T. WILLIAMS and R. L. M. SYNGE. Cambridge University Press, London and New York, 1949. 103 pp. Price \$1.50.

This monograph contains papers given at a symposium held at the London School of Hygiene and Tropical Medicine on October 30, 1948. Partition chromatography in its modern form has been developed since 1941 mainly by A. J. P. Martin and his group of co-workers. The theoretical aspects of the method are reviewed by Martin. The different factors of phase distribution, adsorption, diffusion, and others make the theoretical treatment of partition-chromatography processes very complicated and difficult. So far, progress has been achieved through experimental rather than theoretical work.

A paper by F. Sanger on the application to the study of amino acids and the structure of proteins, and another by C. E. Dent on the use in amino acid and protein metabolism are the highlights of the symposium. In the same way as Tswett's chromatographic method had its greatest success in its application during the 1930's

to the carotenoid field, so has paper-partition chromatography achieved its greatest triumph in the field of amino acids and proteins. At a time when there is a tendency towards the use of more complicated and refined apparatus, it is amazing to see what English research workers have accomplished with the aid of a few strips or sheets of filter paper. Methods have been developed not only for the qualitative and quantitative determination of minute amounts of amino acids, but the composition of small peptides has been elucidated and a new way has been opened for the study of the chemical structure of proteins. The applications to amino-acid and protein metabolism reported by Dent are of great importance to biological and clinical chemistry.

S. M. Partridge reports the use of partition chromatography for the separation and identification of carbohydrates. In this field very interesting results have been obtained recently with the development of new spraying techniques. These methods are applicable to the qualitative and quantitative analysis of biological fluids.

E. C. Bate-Smith has developed a method for the separation of anthocyanins, flavones, and related plant pigments. Separation and identification of the large number of closely related compounds is only possible with the observation of very strict and elaborate controls; even so, the identification of compounds is attended with unusual difficulties, and in the opinion of this reviewer the method in its actual form is not suitable for general use. Separation of colorless hydroxylated benzene derivatives is interesting and promising for the study of plant extracts. C. Rimington discusses briefly the separation of porphyrins into classes according to the number of carboxyl groups. Separation of isomers has not yet been achieved.

R. S. Elsdon describes the quantitative separation and estimation of small amounts of mixtures of lower fatty acids and of physiologically important di- and tricarboxylic acids. These methods need also to be perfected before they can be used by the general worker in the biological field. A beginning has been made in the separation of purines, pyrimidines, and related compounds.

A review by A. A. Levi of methods using a stationary phase other than pure water deals mainly with the use of buffer solutions and their application to the penicillin problem. A general review of the applicability of the method by R. L. M. Synge concludes the symposium.

This book, written by experts in the field, is of outstanding value for the organic chemist and the biochemist. It is the most up-to-date treatise on the subject and will greatly stimulate the progress of research in the application of chromatographic methods to biological problems.

KARL SCHOEN, Kew Gardens, N. Y.

Streptomycin: Its Nature and Practical Applications. Edited by SELMAN A. WAKSMAN, Ph.D., New Jersey Experimental Station, Rutgers University. The Williams & Wilkins Co., Baltimore, Md., 1949. ix + 618 pp. Price \$10.00.

The opening words of the preface read: "Probably no other drug in the history of medical science has had such a phenomenal rise as streptomycin."

This is probably the only sentence in the book which might well have been omitted. Nothing can happen to streptomycin now which will remove Waksman from his place among the giants in the field of antibiotics. But rockets go up with a phenomenal rise. And rockets come down. There must be very few scientists at this moment who

are actively planning to supplant penicillin. But there are many who see visions which would relegate streptomycin to the history books.

Nevertheless, it is true to say that streptomycin, because of its limitations, because of its failings, because of the hopes which have not quite been fulfilled, has added more to our knowledge than penicillin has done, and has stimulated investigations in the field of biochemistry and medicine in a way which must bring outstanding rewards in the future.

Fifty-eight eminent contributors have co-operated in presenting the cream of present information on the subject. It is almost unbelievable that so much could have been accomplished in the space of six years since streptomycin was discovered. Today a major industry is in steady production, and throughout the world medical men are confidently recognizing conditions where streptomycin will succeed and where it will fail.

The book deserves to be, and indeed cannot fail to be, widely read. Many of the chapters are a masterly and dispassionate summation of the available information.

Because of the care and honesty with which the weaknesses of streptomycin are indicated, it would be possible, by quotation apart from context, to add considerably to the alarm and despondency which have frequently been caused by injudicious statements in the popular press. But no just critic can fail to read that streptomycin is a sharp weapon of defense, and used properly can accomplish great things.

The contributions are divided into four main sections. The first section, dealing with microbiological and chemical aspects, includes a carefully balanced review of the nature and nutrition of *Streptomyces griseus* by Waksman himself, complemented by a short paper by Savage on strain selection. Both authors discuss the problem of actinophage, on which considerable progress has been made since this book has been prepared. It is doubtful now, for example, if Waksman would still recommend his third method for selection of streptomycin-producing strains, by exposure to a specific actinophage; Savage is, in fact, critical of this technique.

The chapter by Tishler on production of streptomycin gives a clear picture of the deep-culture process, although one unfortunate sentence may lead a harassed translator behind the Iron Curtain to believe that liquid air is used in fermentation. The portion on streptomycin recovery, although written in great detail, leaves the reader in some doubt as to which methods are considered commercially practicable. The fundamental steps are well described, and it is perhaps unfair to expect specific indications at this stage in the history of a new and competitive industry.

Brink and Folkers, in their review of the chemistry of streptomycin, have provided the most concentrated chapter in the book. It will be followed in detail only by the advanced organic chemist, but no reader can fail to admire the relentless study of the degradation products of the streptomycin molecule, which has led to so complete a picture in so short a time.

Dr. Henry Welch completes the first section with an authoritative chapter on chemical and biological assay.

The second section contains nine papers on the antibacterial and pharmacological properties of streptomycin. The chapters are very closely related, and it is perhaps inevitable that certain items of original work are repeatedly discussed by the various authors. The section is, however, of profound interest, and provides a great deal of food for thought. Particularly readable is the chapter by Feldman and Karlson on

experimental tuberculosis. This is followed by two chapters on the development of bacterial resistance which clearly indicate the problems to be faced in the clinical use of streptomycin. There is little doubt that with most of the pathogens studied, developed streptomycin resistance is long-enduring, if not permanent. The transmission of such strains has obvious dangers.

The chapters on mode of action by Henry and Hobby, and on synergism by Thatcher, merit considerable attention. There is every hope that further studies in these fields may lead to safer and more effective use of streptomycin in combination with other therapeutic agents.

Rake and Donovick deal very clearly and systematically with the absorption and secretion of streptomycin. Pharmacology is presented by Molitor. He examines toxic effects in considerable detail, and, in view of the alarming statements which have been made from time to time, it is fair to quote the well-judged remarks in his first paragraph:

"It is one of the safest drugs known. No deaths attributable to the administration of pure streptomycin by the usual parenteral route have been reported; and the neurotoxic side reactions that occur after prolonged administration of large doses do not imperil the life of the patient, although they constitute a major obstacle to continued therapy. . . ."

The third section of the book is by far the most extensive, and reviews the clinical uses of streptomycin in a series of twenty-six papers. Some of these are necessarily short. With some clinical conditions, streptomycin is obviously not the answer, and no great time need be spent in presenting the evidence. Certain chapters tend merely to catalog reported studies of a particular disease, and more discussion would have been welcomed.

The best chapters represent the basis on which future therapy will be built and will be repeatedly studied both by the specialist and the general practitioner. The chapter on the treatment of tuberculosis in man, by Walker, Hinishaw, and Barnwell, is outstanding, and the authors must be congratulated on their presentation of the information. One would recommend the reader to study their summary on pp. 315-18 before reading any other part of this section.

Again, the chapter by Dr. Hattie Alexander on non-tuberculous meningitis is a clear demonstration of one of the most valuable uses of streptomycin, and of its application in combined therapy.

Meyer and Quan's report on plague is exciting reading even to the layman. That a disease which has been kept at arm's length in Europe and North America only by perpetual vigilance should now be confronted with a weapon like streptomycin is indeed a triumph.

In general, it may be said that meticulous care has been taken throughout the clinical section to present the available knowledge without bias, without overemphasis, either on the accomplishments of streptomycin or the dangers inherent in its use.

The fourth section, dealing with miscellaneous uses of streptomycin, is relatively short, and has merely been included to ensure that the present volume covers the whole field of known work on streptomycin. There are three chapters on veterinary practice; a short chapter on specialized applications in agriculture, chiefly concerned with seed treatment; and a study of the use of antibiotics, including streptomycin.

in the isolation of viruses and other microorganisms. This particular chapter might well have been included in the first section, where Heilman and Rake have also discussed particular aspects of the same subject.

Waksman is to be thanked for undertaking the heavy editorial duty necessary for the presentation of such a representative series of responsible documents. The arrangement has been admirably planned. No one interested in streptomycin—or in antibiotics generally—will be able to omit this book from his library.

J. J. H. HASTINGS, Liverpool, England

Nutritional Data. By HAROLD A. WOOSTER, JR. and FRED C. BLANCK, Heinz Nutritional Research Division, Mellon Institute, Pittsburgh, Pa. H. J. Heinz Co., Pittsburgh, Pa., 1949. vi + 114 pp. Distributed gratis.

Rapid developments in the field of nutrition have necessitated a complete rewriting of "Nutritional Charts for Medical and Other Specialists" (1934) which the present volume supersedes. The material on vitamins, essential elements, and intermediary metabolism has been modernized, a chapter on proteins has been added, the tables on food composition have been reassembled, with recent data added, and the format of the book has been improved.

Summaries are presented under the following chapter headings: Vitamins; The Essential Elements; Proteins and Amino Acids; the Availability of Nutrients; Signs and Symptoms of Malnutrition; Metabolism and Action of Foods; Human Nutritive Requirements; Planning Diets for Good Nutrition; Tables of Food Composition and Nutritive Value; Nutritional Activities of the H. J. Heinz Company; and Suggestions for Further Reading.

The reviewer questions whether arachidonic acid is crystalline (p. 8), whether it is not already proven that "essential fatty acids" are biologically synthesizable (p. 8), whether the International and U.S.P. units of vitamin A and vitamin D are equal (p. 26), and whether the Krebs equations for the metabolism of carbohydrates are now acceptable (p. 52). He suggests that a section on the metabolism of fats in muscle tissues might have been added (p. 54). The authors state that the figures in the food composition tables represent "averages of other tables, which in turn are also averages" (p. 73). This is not a desirable procedure for it tends to magnify and perpetuate errors; furthermore, the values for one food are generally pieced together from several sources. There is an urgent need for a complete re-evaluation of the composition of the foods in the U. S. dietary by across-the-board analysis.

Wooster and Blanck have succeeded in presenting a brief, accurate, and up-to-date summary of current nutritional knowledge. Nutritionists, clinicians, nutritional biochemists, and teachers and students of nutrition in schools and colleges will find this little volume excellent for reference.

ROBERT S. HARRIS, Cambridge, Mass.

Haemoglobin, A symposium based on a Conference held at Cambridge in June 1948 in memory of Sir Joseph Barcroft. Editors: F. J. W. ROUGHTON and J. C. KENDREW. Butterworth Scientific Publications, London, and Interscience Publishers, New York, 1949. xii + 317 pp. Price \$8.50.

The book opens with short biographical tributes written by eight eminent physiologists who were closely associated with Barcroft at various stages of his scientific life, from the time he began, under Langley, his first studies on blood gases (1897) to the

memorable day in spring 1947, when sudden death overtook him in the midst of active work. The tributes are followed by 28 papers by specialists which form a kind of "Recent Advances in Studies on Hemoglobin." Most papers, however, extend far beyond the scope of ordinary review articles as they contain also a great deal of new and previously unpublished experimental evidence.

The papers are arranged according to subject, in seven groups. The subject of *reactions with oxygen and carbon monoxide* is introduced by Drabkin, to be followed by articles of Haurowitz, Pauling, Wyman, and Roughton, each representing a different and original approach to the all-important question as to why hemoglobin should be able to combine without changing the valency of its iron. The papers on *amino acid composition*, by Tristram, Ross-Fanelli, de Duve, and Potter and Sanger, offer a considerable amount of new information on the structural differences between various hemoglobins, and between hemoglobin and myoglobin. The topic of *X-ray investigations of hemoglobin* is introduced by Kendrew and Perutz in a general article on the application of X-ray crystallographic techniques. This section of the book is particularly valuable in view of the remarkable achievements of X-ray crystallography in the field of structural studies on hemoglobin. *Physicochemical properties of the blood pigment* such as osmotic pressure, solubility, and the ultraviolet spectral adsorption, are discussed by Adair, Gutfreund, and Jope. An account of the various *differences between adult and foetal hemoglobin* is given by Jonxis, Jope and O'Brien, and Karvonen. The *comparative biochemistry*, particularly in relation to chlorocruorin and hemocyanin, forms the subject of the papers by Fox, Davenport, and Wolvekamp; the paper by Fox includes a report, the first of its kind, on the simultaneous occurrence of chlorocruorin and hemoglobin in the blood of one animal. Other *biochemical and physiological aspects* include two articles on methemoglobin; one by H. Barcroft, Gibson and Harrison contains a study of a rare case of idiopathic methemoglobinemia in two brothers (40% of the total blood pigment present as methemoglobin) which responded extremely well to treatment with ascorbic acid; Ramsay's article on the other hand, deals critically with the methods used at present for the detection of traces of methemoglobin in the normal blood. The recent progress in studies on the biosynthesis of hem is reviewed by Rimington. In the same section, Vannotti describes his contributions to the problem of the disturbance of hemoglobin synthesis in lead poisoning.

Hemoglobin played a major rôle among Joseph Barcroft's manifold scientific interests in connection with his studies on the respiratory function of the blood, on high altitude, on spleen, on foetal physiology. Nothing would have pleased him more than to be able to read a book which covers divers aspects of hemoglobin in such a comprehensive manner.

T. MANN, Cambridge, England

Recent Progress in Hormone Research. Vol. IV. Edited by GREGORY PINCUS. Academic Press, Inc., New York, N. Y., 1949. 529 pp. Price \$8.80.

This volume includes papers presented at the Laurentian Hormone Conference held at Franconia, New Hampshire, in September 1948. It is divided into four sections. Section I includes five chapters concerning steroid hormone metabolism *in vivo* and *in vitro*. Marrian discusses some aspects of progesterone metabolism and points out the future lines of research. The metabolism of estrogens is presented by Heard and

Saffran and by Segaloff. The recent investigations of Samuels and his collaborators on the metabolism of androgens *in vitro* using liver and kidney tissues are summarized by Samuels.

In Section II, five chapters are devoted to the role of hormones in tissue and body metabolism. It opens with an article by Leatham on the antihormone problem with special reference to the antigonadotrophins. White presents the data which have been obtained by himself and his co-workers in studies of the effects of adrenal cortex, thyroid, and growth hormones on the fasting metabolism of mice. The author emphasizes the control of these hormones on the mobilization of body nitrogen. A similar discussion of this problem was also given by the reviewer in the 1947 Conference.

The contribution on "The Alterations in Metabolism Incident to Administration of Insulin, Adrenalin, and Thyroid Substances, Studies with the Aid of Isotopes," written by Stetten, is of special value, as it is increasingly evident that the tracer studies are becoming one of the important tools in the elucidation of the mechanism of hormonal action. The author outlines the methods and some results which have been obtained from the application of deuterium to the studies of rates of certain biological processes. In "The Pancreas as the Guardian of the Liver," Best gives the evidence that "insulin, by preventing deposition of excess fat in the liver, by preventing ketosis, by encouraging glycogen formation, and by limiting protein breakdown, acts as a protector of the liver."

Investigators, who are interested in the clinical use of cortisone and adrenocorticotrophic hormone (ACTH), will find the chapter by Thorn and Forsham most useful and instructive. For instance, the procedure for the direct eosinophil count following the administration of ACTH is described in detail as a test for the adrenal cortical function.

It is gratifying to find that the first chapter in Section III presents the usefulness of ACTH as a test for adrenal function in cases of mental disease. Besides the chapter by Pincus, Hoagland, Freeman, and Elmadjian, as just mentioned, this section covers subjects related to the neurohumoral-hypothalamic systems: "Manifestation of Altered Autonomic and Humoral Function in Psychoneuroses" by Glegghorn and Graham, and "Effects of Hypothalamic Lesions on Water and Energy Metabolism in the Rat" by Stevenson.

Section IV contains chapters on thyroid physiology and function. The physiologic reactions of the thyroid stimulating hormone (TSH) are summarized by Rawson and Money. Since this hormone is the only one among the six anterior pituitary hormones which has yet to be isolated in pure form, the authoritative summary of the biologic characteristics of TSH is written from experiments using crude hormonal preparations. As pointed out by the authors, we must await the isolation of the pure hormone before the physiologic behavior of TSH can be accurately understood.

The next two chapters deal with clinical data: "The Metabolism of Iodine in Man as Disclosed with the Use of Radiiodine" by Keating and Albert, and "Radiiodine as a Diagnostic and Therapeutic Tool in Clinical Medicine" by Seidlin.

Like previous volumes, each chapter is followed by a discussion which is one of the most attractive features of the Conference. Sometimes information given by the discussants consists of unpublished data and is informative in nature. Occasionally one may find amusing and poetic passages which convey to the readers the personality

and mannerism of the individual. It is hoped that high lights of this sort will continue to appear in the future volumes.

Since general interest in hormone research has rapidly increased during recent years, there is little doubt that "Recent Progress in Hormone Research" will become very popular with biochemists, physiologists, and medical scientists; however, the price of the present volume may prevent many from placing it in their libraries. Finally the reviewer feels that every effort should be made to publish it not later than six months after the Conference so as to make future volumes even more valuable to the endocrinologists.

CHOU HAO LI, Berkeley, California

Vitamin Methods. Vol. I. Edited by PAUL GYÖRGY, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, Academic Press Inc., New York, New York, 1950. x + 571 pp. Price \$10.

The steady advances being made in the techniques of vitamin assay make it almost inevitable that a new survey of available methods should appear. Volume I is a fairly comprehensive compendium of physical, chemical, and microbiological methods for the determination of the vitamins. The plans for Volume II call for sections on animal experimentation, laboratory diagnosis of vitamin deficiencies, clinical methods, and statistics, and hence between the two it is hoped that the entire field of vitamin assays will be covered. Volume I contains five sections: physical methods, chemical methods, microchemical methods, microbiological methods, and optical instruments. Most of these deal with a general method of assay applied to as many different vitamins as possible.

This method of organization follows the classical pattern of Abderhalden's "Arbeitsmethoden" rather than the more familiar one in which each of the vitamins is discussed more or less thoroughly in turn. There are some advantages to the procedure employed. The authors have been chosen for their familiarity with the type of assay discussed, and at least two of them have personally made significant contributions to the development of these assays: O. A. Bessey on microchemical methods, and E. E. Snell on microbiological methods. The arrangement makes it possible to preface the specific procedures with a general treatment applicable to more than one vitamin. This has been done to some extent in E. T. Stiller's chapter on physical methods, and even more so in E. E. Snell's chapter on microbiological methods, which is divided into sections dealing with lactic acid bacteria, yeasts, and *Neurospora*.

But whether these advantages outweigh the disadvantages inherent in the method of organization is debatable. The book is not as easy to use as it might be, since the methods applicable to a given vitamin, thiamine, for example, may be scattered in four or five different parts of the book. A more serious fault is the absence of any indication of the comparative value of different types of assay for any one vitamin. The authors have made some effort in this direction within their own chapters, *e.g.*, György and Rubin on the chemical determinations of choline or of ascorbic acid, and Snell on the microbiological determinations of niacin. But the investigator who is confronted with the problem of choosing between a physical and a microbiological method for riboflavin, or between a chemical method for choline and one involving *Neurospora*, will have to depend upon his own resources. A short summary chapter

inserted into the next volume evaluating all of the various methods for each vitamin in turn would certainly be helpful.

Perhaps the strongest features of Volume I as a whole are the thoroughness with which many of the procedures are described, sometimes in the exact words of the original authors, the many diagrams and detailed calculations, the frequent references to other methods, and the very extensive bibliography. A short final chapter by E. Hirschberg describes commercial optical instruments common to biochemical laboratories with a helpful bibliography to the published material which ordinarily accompanies new instruments but which usually has disappeared by the time one needs it.

C. A. BAUMANN, Madison, Wisconsin

Methods of Vitamin Determination. By B. CONNOR JOHNSON, Associate Professor, Division of Animal Nutrition, University of Illinois. Burgess Publishing Co., Minneapolis, Minn., 1948. iv + 109 pp. Price \$3.00.

Teachers, students, and vitaminologists will be interested in Dr. Johnson's laboratory manual but each for quite different reasons. The teacher will be pleased at the opportunity of getting in one book such a vast amount of well-organized vitamin methodology which can be adopted wholly or in part for his own classes.

His students who master the technics described in the manual will be very much in demand in industrial and academic laboratories where chemical and microbiological vitamin assays are performed routinely.

The expert in vitamin technology will be interested in the selection Dr. Johnson, one of their members, has made of all the available analytical procedures. The vitamin technologist probably will also comment on some of the following ambiguities: On p. 11 the explanation of the slope-ratio method for calculating results of microbiological assays should be amplified; *e.g.*, how closely must a_s and a_i agree before the results of the assay are accepted as valid. Also, the meaning of potency-ratio, R , should be clarified. On p. 15 the amount of light *transmitted* is measured in colorimetric and spectrophotometric analyses. On p. 20 "exit slit" not "exist." On p. 31 *castrated* rats should be specified for bioassay by the vaginal smear method. Measurement of liver storage of vitamin A is a rapid and acceptable method of bioassay which might at least have been mentioned. The U.S.P. standard for vitamin A has been crystalline vitamin A acetate (not cod liver oil) dissolved in refined cottonseed oil since January 1, 1948. On p. 34 the value for $E_{1\text{cm}}^{1\%}$ at 622 $m\mu$ for vitamin A is incorrectly stated as 3990. This value should have been 4390. [*J. Am. Chem. Soc.* 65, 2479 (1943).] On p. 35, the reference to gelatin capsules containing vitamin A for standardization should be to the U.S.P. Reference Standard in capsules each of which contains 2500 U.S.P. units of vitamin A and which may be obtained from the U. S. Pharmacopoeia, 4738 Kingsessing Ave., Philadelphia 43, Pa. The $E_{1\text{cm}}^{1\%}$ at 328 $m\mu$ is used correctly on pp. 17 and 37 as 1750, but appears as 1780 on p. 36.

The AOAC procedure for assaying for vitamin D content is not primarily a pass-or-fail type test since it permits a fairly precise estimate of potency to be made. On p. 42, the direction "a quantity of fish liver oil sufficient to yield approximately 25 mg. or more of the vitamin is boiled . . ." would mean about 1 million units of vitamin D (25 \times 40,000 units/mg.) must be used for each test—this would be a tremendous volume of most vitamin D oils. Twenty-five micrograms probably is meant.

A newer and more quantitative bioassay procedure for vitamin E than that mentioned on p. 44 is described in *Biol. Symposia* 12 (1947).

On p. 48 the two assay diets for thiamine determination need not be detailed separately since they would be essentially identical if the 2nd diet were corrected—its ingredients total only 90%.

The directions on p. 57 for compounding the riboflavin-deficient diet are not clear regarding the pure B-vitamins listed. Should these be used in addition to the 80 mg. of rice-polish extract?

The formula for the lactone of pantoic acid on p. 78 shows a couple of carbon atoms and an oxygen atom with a valence of 3 and another carbon with a valence of 5. Minor errors in spelling occur: on p. 33, "hydroxide"; p. 49, "Experientia"; p. 96, "Lucky," and p. 107, "guanine."

On p. 65 why should the modification of method, which gives the *higher* value for nicotinic acid, be used?

There is a general lack of consistency in using formulas, symbols, and terms. Also, in the Outline of Lectures, which serves as a table of contents, no mention is made of Chap. 20 "Microbiological Determination of Amino Acids" (vitamin ?), and conversely the subject of unsaturated fatty acids is listed among the lectures but is not discussed in the text.

In spite of these criticisms—mostly of minor significance—"Methods of Vitamin Determination" will probably find rather wide usage particularly in subsequent, corrected editions, revised to include recent advances. It will have few competitors since hardy authors, such as Dr. Johnson, who are willing to tackle so wide and constantly changing a subject as the analysis of vitamins are relatively scarce.

PHILIP L. HARRIS, Rochester, N. Y.

Biochemical Evolution. By MARCEL FLORKIN, University of Liège, Belgium. Edited/translated, and enlarged by Sergius Morgulis, College of Medicine, University of Nebraska. Academic Press Inc., New York, N. Y., 1949. vi + 157 pp. Price \$4.00.

Since the publication in 1903 of "Vergleichende chemische Physiologie der niederen Tiere" by Otto von Fürth, few attempts have been made to collect together the now numerous and widely scattered papers which form the literature of comparative biochemistry. Professor Florkin's "Biochemical Evolution" has achieved a skillful synthesis.

Necessarily, Professor Florkin's book, which presents the thesis that "evolution and the classification of animals can be considered from a biochemical viewpoint," presents a great mass of data from many different fields of biochemistry, the orderly presentation of which must have been a serious problem in itself. It is not an easy book to read; but the reading is well worth the effort it entails.

It is regrettable but perhaps inevitable that much of the charm so characteristic of the original French edition should have been lost in translation, but this loss is to some extent compensated by additions made by the translator. New material has been added at many points, occasionally at the expense of the continuity of the original text, but there is one particularly valuable addition in the form of a bibliography containing almost 300 references which provide a skeleton key to the literature. For this we owe thanks to Dr. Morgulis, the editor and translator.

The book is attractively printed and is well bound; there are, however, a number of typographical errors. Although few of these are of any serious character, Table IV contains one gross error in that the presence of both arginine and creatine phosphates is indicated in Ophiuroidea: this error does not appear in the original text, and arginine phosphate has never, to the reviewer's knowledge, been detected in representatives of this class. Some statements which have been faithfully translated from the original would have been the better for editorial revision in the light of progress made since the first French edition appeared in 1944. For instance we find a statement to the effect that "the yellow enzyme takes care of fatty acid metabolism," while the breakdown of adenosine triphosphate in glycolysis is still reported as yielding adenylic acid, rather than adenosine diphosphate.

A regrettable feature of the book is the occasionally teleological attitude displayed by the author. Perhaps this is unintentional, but it is disturbing to find such statements as "Evolution frequently utilizes an already existing substance for a new function by creating a new system of biochemical receptors for its action," and, "It is most probable that the accumulation of ammonia in the internal medium was the reason for the production of a much less soluble substance like uric acid . . ." (Reviewer's italics.)

These are however only blemishes. The book, as a whole, is a worthy presentation of a point of view which is still novel enough to need forceful treatment, and it is one which every biochemist should read with care, and which many other biologists might read with profit. There is much of interest between the covers and not a little that is probably fundamental.

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Errata

In the article by Martin, Wilson and Burris entitled, "Citric Acid Formation from $C^{14}O_2$ by *Aspergillus niger*," which appeared in Volume 26, Number 1, p. 103, the value for initial citric acid should read 152.1 instead of 192.7.

In the Letter to the Editors by Ma and Fontaine entitled, "Identification of the Sugars in Crystalline Tomatin," which appeared in Volume 27, Number 2, p. 461, *tomatin* should read *tomatine* throughout.

Serum Iron Levels of Adolescent Girls and the Diurnal Variation of Serum Iron and Hemoglobin

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INTRODUCTION

Few studies of the iron content of the serum of adolescent girls have been made; in a search of the literature, only three were found (Table I). The highest serum iron level was reported by Vahlquist (18) who observed a mean of 122 $\mu\text{g.}/100\text{ ml.}$ for 23 girls, 14–15 years of age. A mean somewhat lower than that, 107 $\mu\text{g.}$, was reported by Dahl (4) for 10 girls, 13–16 years of age. Johnston (10) observed mean values of 46,² 62, and 72 $\mu\text{g.}$, respectively, for each of three girls, 13 and 14 years of age, upon each of whose serum determinations had been made in the late afternoon 11 times over a period of 15 weeks.

The purpose of the present study was not only to obtain additional information regarding normal serum iron levels in adolescent girls, but also to find whether or not the level for them was lower than the level for women and whether or not the serum iron level was affected by the habitual intake of dietary iron. Since adolescent girls have a greater need for iron than subjects at any other age, if the iron intake has an effect on the iron content of the serum, the effect should be evident in a shorter time for them than for older or younger subjects. That the serum iron level does not respond rapidly to the dietary intake of iron, even for adolescent girls, was demonstrated by Johnston (10) who studied the serum iron levels of three subjects while their intake was varied. The serum iron levels of the girls did not fall significantly over

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² This subject had unusually large menstrual losses.

TABLE I

Serum Iron Values Found in the Literature for Girls 13 to 16 Years of Age

| Investigator | Year | No. of subjects | Age | No. of determinations per subject | Average serum iron | Range | Difference |
|----------------|------|-----------------|------------|-----------------------------------|------------------------|------------------------|----------------|
| | | | <i>yr.</i> | | $\mu\text{g./100 ml.}$ | $\mu\text{g./100 ml.}$ | $\mu\text{g.}$ |
| Vahlquist (18) | 1941 | 23 | 14-15 | 1 | 122 | 65-189 | 124 |
| Johnston (10) | 1947 | 3 | 13-14 | 11 | 60 ^a | 37-93 | 56 |
| Dahl (4) | 1948 | 10 | 13-16 | 1 | 107 | 83-136 | 53 |
| This study | 1949 | 27 | 13-16 | 3 | 77 ^a | 39-127 | 88 |

^a These determinations were made between 4:00 and 5:00 p.m.

a 9-week period on an intake of 4 mg./day nor did it rise over a period of 12 weeks on an intake of 9 or 11 mg. There is the possibility, however, that over a longer period of time, the serum level may respond to the dietary intake of iron. To find the effect of a given dietary intake for a longer period of time, the history of the dietary intake of these subjects was investigated.

PROCEDURE

The subjects, 27 girls 13-16 years of age, all of whom had passed the menarche, were students in a junior high school and came from families of widely different economic levels. In anticipation of finding subjects who had been living on low iron intakes, eight were selected from families with very low incomes. Physical examinations were not made except for six girls (Nos. 22-27) (Table II) who were subjects in a rigidly controlled iron balance study; they were judged to be in good health.

For each subject, serum obtained from blood samples taken at three different times was analyzed. For 21 subjects (1-21), the serum was obtained from three different venipunctures made within 1 week; for six subjects (22-27) blood samples were taken three times during a 9-week period at approximately 3-week intervals. In all cases, approximately 10.5 ml. of blood was withdrawn between 4 and 5 p.m. after the subject had been sitting quietly for half an hour to eliminate the possible effects of exercise on the hemoglobin concentration and red blood cell counts. Although samples taken under fasting conditions were desired, the difficulties of obtaining samples from school children before breakfast in the morning were so great that the after-school hour was employed instead. The experiment was, however, preceded by a sup-

plementary study on adult women from whom samples were taken before breakfast and in the late afternoon in order to find the magnitude of the difference, if any existed.

TABLE II

Mean Values for Three Determinations of Serum Iron, Hemoglobin, Red Blood Cell Count, and the Estimated Dietary Iron Intake of 27 Adolescent Girls

| Subject | Age | Serum iron | | Hemoglobin | Red blood cells | Iron intake |
|-------------------|-----|------------------------|------------------------|---------------------|------------------------------|--------------------|
| | | Mean | Range | | | |
| | yr. | $\mu\text{g./100 ml.}$ | $\mu\text{g./100 ml.}$ | g./100 ml. | $\times 10^6/\text{cu. mm.}$ | mg./day |
| 1 | 16 | 66 | 51-88 | 14.0 | 4.49 | 7 |
| 2 | 14 | 85 | 62-98 | 14.9 | 4.93 | 6 |
| 3 | 15 | 78 | 66-93 | 14.1 | 4.48 | 13 |
| 4 | 15 | 63 | 42-89 | 14.3 | 4.15 | 7 |
| 5 | 13 | 91 | 81-105 | 14.5 | 4.68 | 15 |
| 6 | 14 | 69 | 65-73 | 13.3 | 4.59 | 10 |
| 7 | 14 | 98 | 61-121 | 13.2 | 3.86 | 8 |
| 8 | 15 | 76 | 70-86 | 13.1 | 4.26 | 15 |
| 9 | 15 | 86 | 76-98 | 14.2 | 4.69 | 9 |
| 10 | 14 | 54 | 39-81 | 12.0 | 4.46 | 11 |
| 11 | 14 | 71 | 45-100 | 12.7 | 4.34 | 11 |
| 12 | 14 | 74 | 59-83 | 13.1 | 4.24 | 13 |
| 13 | 14 | 51 | 42-60 | 14.0 | 5.03 | 9 |
| 14 | 14 | 87 | 77-105 | 14.6 | 4.92 | 9 |
| 15 | 14 | 93 | 58-125 | 15.0 | 4.90 | 11 |
| 16 | 14 | 92 | 58-127 | 13.7 | 4.73 | 9 |
| 17 | 16 | 87 | 79-91 | 13.6 | 4.36 | 9 |
| 18 | 14 | 79 | 68-84 | 14.5 | 4.69 | 10 |
| 19 | 15 | 92 | 83-101 | 12.9 | 4.29 | 6 |
| 20 | 14 | 82 | 73-87 | 13.8 | 4.80 | 8 |
| 21 | 15 | 64 | 50-77 | 14.4 | 4.81 | 9 |
| 22 | 14 | 97 | 92-105 | 13.7 | 4.81 | 9, 12 ^a |
| 23 | 14 | 80 | 76-86 | 13.5 | 4.45 | 9, 12 ^a |
| 24 | 13 | 65 | 44-76 | 13.7 | 4.54 | 9, 12 ^a |
| 25 | 14 | 57 | 50-64 | 13.2 | 4.35 | 9, 12 ^a |
| 26 | 13 | 78 | 74-85 | 14.9 | 4.83 | 9, 12 ^a |
| 27 | 13 | 71 | 70-72 | 14.1 | 4.49 | 9, 12 ^a |
| Mean | | 77 | | 13.8 | 4.56 | |
| S.D. ^b | | 13 | | 0.7 | 0.27 | |

^a The dietary intake was 9 mg./day when samples 1 and 2 were taken and 12 mg./day when sample 3 was taken. Each of these subjects had a history of good food habits.

^b Standard deviation.

For the study of diurnal variation, 15 women between the ages of 24-47 years served as subjects. All were graduate students or faculty members in a food and nutrition department of a college. Samples of venous blood were taken under fasting conditions between 7:30 and 8:00 a. m. and between 4:30 and 5:30 p. m. Before samples were taken, any subject who had been performing active work such as outdoor walking or laboratory work rested for 0.5-0.75 hr., a precaution observed to eliminate the effect of exercise on hemoglobin concentration.

Hemoglobin was determined by the method of Evelyn (6). Red cell counts were made in duplicate on blood from each of two pipets.

For the determination of serum iron 2 ml. of blood serum was pipetted into a 15-ml. centrifuge tube. To this, 1.5 ml. of glass-distilled water was added and the protein was precipitated as described by Kitzes *et al.* (11). The supernatant liquid was decanted into a 10-ml. volumetric flask and the iron content was determined by the 1,10-phenanthroline method of Saywell and Cunningham (15). Recoveries of 94, 99, and 101% were obtained from 2-ml. portions of serum to each of which 1 μ g. of iron had been added; these analyses were made on three different lots of serum on three different days. As a further check on the method, determinations were made on samples of different sizes: for 2 ml. of serum, the mean of seven samples was 0.73 μ g./ml.; for 3 ml. the mean was 0.72 μ g.

People are apprehensive when blood samples are to be taken, even though attempts are made to minimize the strain of the situation. Some people are more apprehensive than others and a given person may be more apprehensive at one time than another. To gain evidence as to whether or not stress or excitement stemming from apprehension raises the hemoglobin level, determinations were made of blood sugar concentrations, and blood pressure and pulse counts were taken for 13 girls (Nos. 9-21).

For the purpose of obtaining information regarding the customary dietary intake of iron, 21 girls (1-21) were asked to keep a 3-day food intake record. In addition, each of these girls was interviewed by a nutritionist concerning her dietary history. To obtain information about the habitual intake, the approximate iron content of their diets was calculated (1,3) from the records. The six girls who were on the iron balance experiment had a history of good food habits; no food-intake records were kept by them when they were living at home on their habitual diets.

RESULTS AND DISCUSSION

The mean serum iron values for 27 girls, whose serum values were determined in duplicate on three different days, between 4:00 and 5:00

p. m., was $77 \pm 13 \mu\text{g.}/100 \text{ ml.}$ (Table II); single values ranged from 39 to $127 \mu\text{g.}$ The mean found in this study is lower than those of $122 \mu\text{g.}$ and $107 \mu\text{g.}$, reported by Vahlquist (18) and Dahl (4), respectively, for girls of the same age: the lack of agreement is not surprising since serum iron values for subjects at other ages reported by different investigators vary widely, also.

The lack of agreement in the serum iron values found by different investigators may be attributed to one or more factors: the method employed for analysis, actual differences in the amount of iron in the serum of the groups studied, and differences in the time of the day when the samples of blood are withdrawn. The method used in this study differed in some respects from that used by Vahlquist (18) and Dahl (4). The serum iron level of the subjects of this study may not have been high; while no subject had an obvious physical defect which might have affected the blood picture, no physical examination was made to screen out girls who were in poor health. Vahlquist and Dahl gave no information regarding the selection of their subjects. In this study the blood samples were withdrawn between 4:00 and 5:00 p. m. Neither Vahlquist nor Dahl reports the time of day when the samples were taken.

In the study of the diurnal variation of serum iron in women which preceded the study on adolescent girls, the iron content of the serum drawn late in the afternoon was found to be lower than that drawn in the morning under fasting conditions. The mean difference for 16 pairs of determinations was $23 \mu\text{g.}/100 \text{ ml.}$ (Table III). The *F* test showed this difference to be significant at the 1% level. This change in the serum iron during the day is similar to a fall of $31 \mu\text{g.}$ found by Høyer (8) who studied six women for 7 days from 8:00 a. m. to 4:00 p. m., and Hemmler (7) who studied 23 women for 1 day. If $23 \mu\text{g.}$ is added to the mean value observed in the serum of the girls of this study the resulting value of $100 \mu\text{g.}$ approaches that of $107 \mu\text{g.}$ reported by Dahl (4).

The mean value of $77 \mu\text{g.}/100 \text{ ml.}$ for the adolescent girls of this study is approximately the same as that of $73 \mu\text{g.}$ (Table III) for the 16 determinations on adult women. For both groups the blood samples were withdrawn between 4:00 and 5:00 p. m. and the same techniques and methods of analysis were used. Thus, although adolescent girls require much iron to meet the needs for growth and cover the losses in the

TABLE III
Diurnal Variation in Hemoglobin and Serum Iron

| | Hemoglobin | | | Serum iron | |
|------------------------|--------------------|-------------|------------------------|--------------------|-------------|
| | a. m. | p. m. | | a. m. | p. m. |
| | g./100 ml. | g./100 ml. | | μg./100 ml. | μg./100 ml. |
| Mean of 23 experiments | 13.9 | 13.7 | Mean of 16 experiments | 96 | 73 |
| S. D. ^a | 0.7 | 0.6 | S. D. ^a | 28 | 28 |
| Sources of variation | d. f. ^b | Mean square | | d. f. ^b | Mean square |
| Time | 1 | 1.1756 | | 1 | 8789.0625 |
| Experiments | 22 | 1.6211 | | 15 | 1860.2167 |
| T. E. | 22 | 0.1968 | | 15 | 866.6958 |
| Duplicates | 46 | 0.1096 | | 32 | 29.9648 |

^a Standard deviation.

^b Degrees of freedom.

menses, their blood serum contains no less iron than that of adult women.

Serum iron levels of 50 and 60 μg./100 ml. have been suggested by Moore *et al.* (14), and Burch *et al.* (2), respectively, as the lower limits of the normal range. They have not specified whether or not these values apply for every hour of the day. The lowest mean serum iron levels observed in this study were those of 54, 51, and 57 μg. for subjects 10, 13, and 25, respectively. These girls appeared to be healthy and no explanation for the low values was found. Subject 25, who participated in the iron balance experiment and was one of the six subjects under close supervision for 9.5 weeks, possessed abounding vitality, yet her average serum iron level was below 60 μg. She was the most active member of the group of girls. This is an interesting observation in view of the fact that Delachaux and Ott (5) found that exercise lowered the serum iron level.

Wide variability was noted in the values found in this study (Table II). The finding is in agreement with that of other workers in the field. Vahlquist (17) aptly stated that "serum iron under physiological conditions has a great tendency towards variation; one is tempted to say surprisingly great." Because of the wide divergence of values usually found in the three values for any one subject, the reliance which can

be placed on the analysis of only one sample of serum as a representative value for a given subject is questionable. Subject 11, for example, had serum iron levels of 45, 100, and 67 $\mu\text{g.}/100\text{ ml.}$ in the three samples taken. Had only the first value been available for this subject, she would have been considered as deficient in iron according to the criterion advocated by Burch *et al.* (2), who considered a value less than 60 $\mu\text{g.}/100\text{ ml.}$ as one rarely found in a well-nourished individual. In view of this variability fewer than three values would appear to be insufficient to give a representative value for a given individual.

One of the objects of this study was to find whether or not the serum level reflects the habitual dietary intake of iron. The estimated intakes ranged from 6 to 15 mg./day (Table II). The correlation between iron intake and serum iron was 0.00; subjects with high dietary intakes did not necessarily have high serum iron levels nor did subjects with low intakes have low serum iron levels. Several explanations may be offered for this lack of correlation. Intake records do not furnish accurate estimates of dietary intake, especially for iron, because of the possibility of contamination. Nevertheless most of the low intakes were probably relatively low. Stored iron may have been a factor; although the deficiency of iron in these subjects was probably not severe enough to remove from storage iron which had been deposited several months before, iron deposited recently during short periods of high intake not revealed by the diet history may have been withdrawn. Apparently none of the subjects had been receiving so little iron in her diet that the serum iron was lowered below 50 $\mu\text{g.}/100\text{ ml.}$ Since a large proportion of the children came from homes of the lowest income in the community, apparently few children receive so little iron in their diets that the serum iron is reduced below that level. Possibly when the diet supplies enough iron to permit a serum iron level above 50 $\mu\text{g.}/100\text{ ml.}$, the effect of the dietary intake of iron is obscured by the effect of other physiological mechanisms.

The blood picture as measured by hemoglobin concentration and red blood cell count was satisfactory for the subjects. The mean hemoglobin value for duplicate determinations for all the adolescent girls was $13.8 \pm 0.7\text{ g.}/100\text{ ml.}$ of blood and ranged from 12.0 to 15.0 g.; the mean red blood cell count for duplicate determinations on blood from each of 2 pipets was $4.56 \pm 0.27 \times 10^6/\text{cu. mm.}$, and ranged from 3.86 to $5.03 \times 10^6/\text{cu. mm.}$ The value observed for the hemoglobin for these subjects was in close agreement with that found by Wiehl (19) in a study of 98 girls, 13–16 years of age, all of whom came from high-income

families; the value found by Wiehl (19) for the red blood count was slightly higher than that found in this study.

In the supplementary study of the diurnal variation of hemoglobin in women, the mean fall in hemoglobin from morning to late afternoon for 23 pairs of determinations made on blood from 16 women was 0.2 g./100 ml. (Table III). The *F* test showed this difference to be significant at the 5% level. Although the decrease in hemoglobin was of statistical significance, it was so small as to be of little practical importance. This decrease was less than that reported recently (1946) by Wilkins and Blakely (20) who found that the hemoglobin values for blood drawn in the afternoon were considerably lower than those for blood taken in the morning. For a group of 651 children, they found a statistically significant decline in hemoglobin of 0.63 g.; most of the samples were drawn between 9:00 a. m. and 3:00 p. m. They made determinations, also, on blood samples from one group of 10 and another of 11 college girls, and found a mean fall in hemoglobin during a 9-hr. interval of 1.36 and 1.06 g./100 ml., respectively. The findings of the present study are in agreement with those of Smith and Prest (16), McCarthy and Van Slyke (13), and Leichsenring *et al.* (12), all of whom found lower values in the late afternoon than in the early morning; but the difference was so small as to be of little importance. Johnson *et al.* (9) concluded from studies on a group of men that the time of day made no difference.

While stress may raise the hemoglobin value, no evidence of such an effect was obtained in this study on adolescent girls since no consistent relationship existed between the values for hemoglobin and for blood sugar, blood pressure, and pulse count.

SUMMARY

Serum iron levels, hemoglobin concentrations, and red blood cell counts were determined on three different days for each of 27 girls, 13-16 years of age, all of whom had passed the menarche. The mean serum iron value was 77 ± 13 μ g./100 ml.; single values ranged from 39 to 127 μ g. The samples of blood were taken between 4:00 and 5:00 p. m. at which time serum iron values are lower than in the early morning. According to a supplementary study made on 16 women, the mean fall in the iron content of the serum from early morning to late afternoon was 23 μ g./100 ml.

The mean serum iron level of these girls was essentially the same as

that of adult women from whom blood samples were taken at the same time of day. For both groups the same methods and techniques for collection and analysis were used.

The mean hemoglobin value of the blood drawn between 4:00 and 5:00 p. m. from adolescent girls was 13.8 ± 0.7 g./100 ml. of blood, and the mean red blood cell count was $4.56 \pm 0.27 \times 10^6$ /cu. mm. According to a supplementary study on 16 women in which 23 pairs of determinations were made early in the morning and in the late afternoon the mean fall in hemoglobin was 0.2 g./100 ml.

According to estimates from 3-day diet records, the intake of iron varied among the subjects from 6 to 15 mg./day. Diet histories indicated that the 3-day intake was typical of the habitual intake. No correlation was found between the estimated iron intake and the level of iron in the serum.

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The Combination of Organic Anions with Serum Albumin.

VII. Stabilization Against Denaturation by Ultraviolet Irradiation¹

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INTRODUCTION

Many agents, *e.g.*, urea, heat, and ultraviolet light denature proteins, denaturation being indicated by the decrease in solubility of the protein in water and salt solutions of low ionic strength and by changes in other specific properties. In earlier papers of this series (1-5) it has been shown that the salts of various aliphatic and aromatic acids maintained almost unchanged the viscosity and certain other properties of bovine albumin solutions even when the solutions were subjected to heat or urea under conditions which would otherwise effect denaturation. The study reported here was made to determine whether such salts would prove effective in stabilization against denaturation by ultraviolet radiations. The study, made chiefly with sodium caprylate, shows that such salts reduce the extent of ultraviolet denaturation of serum albumin as shown by the development of decreased viscosity and by less marked increases in ultraviolet absorption.

EXPERIMENTAL

Amorphous bovine serum albumin was employed in most of the experiments³ and solutions were prepared as before (4). The particular compounds investigated for their

¹ The painstaking technical assistance of Mrs. J. Legg is gratefully acknowledged. Grants-in-aid from the Rockefeller Foundation and the Cutter Laboratories have made the investigation possible.

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³ The amorphous bovine albumin was provided through the generosity of Dr. J. L. Porsche of the Armour Laboratories.

ability to modify the ultraviolet denaturation of serum albumin were portions of the same lot of chemicals employed in the earlier work (4) and were used as the sodium salts. The viscosity measurements were made with Ostwald viscometers in a 30°C. bath (4). The effect of caprylate upon the ultraviolet denaturation of albumin was investigated in greatest detail, since this compound does not absorb light in the part of the ultraviolet spectrum employed. Aromatic compounds absorbing strongly in the ultraviolet could only be added after irradiation and their use was restricted to exploratory experiments described under Sec. 3 of *Results*.

The lamp used for irradiating the albumin solutions was a low-pressure mercury resonance lamp with 85% of its output at 2537 Å. Some energy is emitted at λ 1849 Å, especially since the lamp used had a quartz envelope. The quantum yield is probably much higher at this wavelength than at 2537 Å, but there was no way available for determining its relative intensity. A 13-ml. sample of a 1% albumin solution 4.5 mm. deep was irradiated in a quartz-covered vessel cooled with a fan which also cooled the arc. The intensity of the lamp determined with a Hanovia Ultraviolet Meter was found to be 99 ergs/mm.²/sec. at the same distance as the arc from the surface of the solution. In the 3-hr. exposure usually used, the dosage was 1,069,000 ergs/mm.² When the solutions were heat-treated, unless otherwise indicated, they were exposed for 1 hr. at 50°C.

The pH of the solution was adjusted to pH 7.4 during the dilution to 0.1% albumin prior to measurement of the ultraviolet absorption. The ultraviolet absorption was determined in the usual manner with a Beckman quartz spectrophotometer.

RESULTS

Viscosity Determinations

Early in the course of the experimental work it was found that the viscosity of an irradiated, 18% albumin solution buffered at pH 7.4 was only slightly different from that of the unirradiated control when short exposures were used; therefore irradiation alone does not markedly alter the viscosity of the solutions. However, when these concentrated albumin solutions irradiated for more than 1.5 hr.⁴ (see Table I, 4th line) were subsequently heated, they turned to clear or slightly turbid gels unless one of the "stabilizer" salts such as sodium caprylate, picrate, salicylate, or mandelate, or acetyltryptophan were present. This suggests the possibility that ultraviolet radiations produce changes in the albumin molecules leading to their aggregation or polymerization upon heating. Therefore, 9% solutions were subjected to the combination of irradiation and heat treatments, but little difference was obtained between the aqueous viscosities of treated and untreated solutions (viscosities being measured for solutions of 3% albumin concentration). It was thought that the change in viscosity resulting from the combination

⁴ Exposures up to 5 hr. were tried.

of one native albumin molecule with another might not be observable because the axial ratio would still be too small after such treatment. However, following denaturation of such treated albumin samples with dodecyl sodium sulfate (DSS) which is thought to unfold the globular molecules, aggregation of albumin molecules would greatly influence the viscosity since the axial ratio of such aggregates might be double that of the unfolded albumin molecule. The DSS would act as an "amplifier" for the change. Therefore the control and the three treated albumin samples were next subjected to denaturation in 2% dodecyl sodium sulfate.⁵

Our surmise proved to be correct as borne out by the data given in Fig. 1 in which measurable differences were found between DSS denatured albumin controls and those subjected to irradiation alone as well as heated after irradiation. Figure 1 presents the relative viscosity *vs.* concentration curves for these experiments. Examination of this figure shows the following: (a) Heating has little effect on the viscosity as shown by the lack of change in the slope of the curve obtained; (b) irradiation alone has increased the viscosity as shown by the change in slope of the viscosity curves obtained after irradiation of albumin solutions; and (c) irradiation followed by heating greatly increases viscosity as shown by the increase in the slope of the viscosity curve.⁶

It now remained to determine whether those compounds which stabilize albumin against heat denaturation will also stabilize it against ultraviolet denaturation. Dodecyl sodium sulfate "amplification" of effects of any treatment on the viscosity of the albumin as described in the preceding experiments was used here as well. Figure 1 summarizes the results of these experiments. It will be observed that (a) caprylate slightly reduces the viscosity of the control as well as of the heated preparation; (b) caprylate stabilizes against the rise in viscosity due to irradiation since the curve for the irradiated preparation containing caprylate practically coincides with the control, whereas without caprylate it is much steeper, indicating that the viscosity rises considerably

⁵ The use of DSS as denaturant is preferable to other substances such as urea, since, as shown by preliminary trials, caprylate and the other compounds investigated have less effect upon the viscosity of albumin so denatured. Any changes in slope of the curves obtained could then be more definitely ascribed to the ability of a "stabilizer" to modify the ultraviolet denaturation.

⁶ Experiments were carried out at pH 6 and 7.5. Only the data for pH 7.5 are given since the differences are more striking than at the lower pH, but the relative order was the same in both cases.

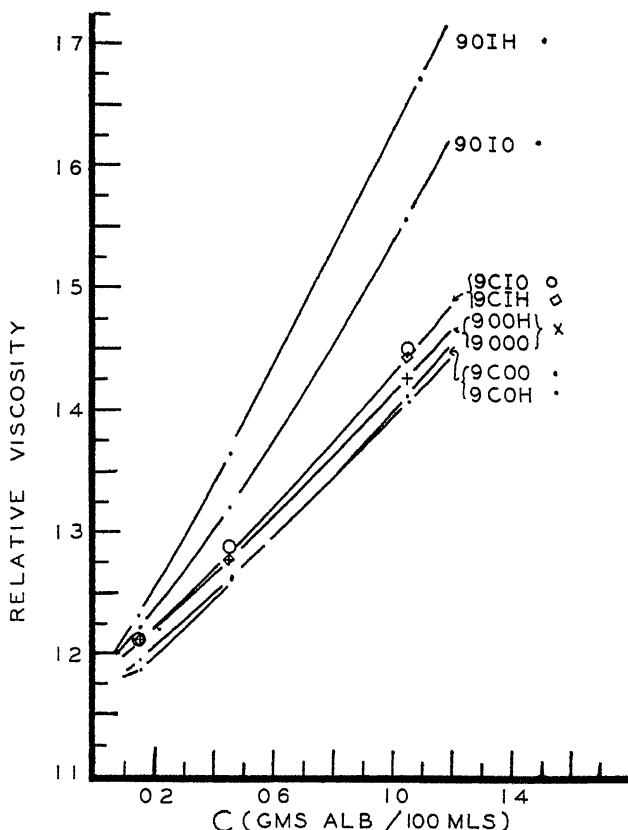


FIG. 1. The influence of ultraviolet radiations or heating on the viscosity developed in albumin-DSS solutions. The code indicates consecutively the treatment to which each solution was subjected; the meaning of each code term is as follows: 9 refers to the use of 9% amorphous bovine serum albumin in 0.05 *M* phosphate buffer at pH 7.5; *I* equals 6 hr. irradiation at a distance of 87 mm. from the face of the arc; *H* means 1 hr. of heating at 50°C., and *C* refers to the presence of 0.05 *M* sodium caprylate throughout the treatment. When a zero is present, that part of the treatment in the sequence was omitted.

Following the indicated treatments the albumin samples were diluted with DSS solution so as to give the final albumin concentrations indicated on the abscissas and a final DSS concentration of 2%. After 24 hr. standing the relative viscosity at 30°C. was determined for each of these mixtures. The relative viscosity values are with reference to the 2% DSS solution.

after the treatment; and (c) caprylate not only stabilizes against this increase in viscosity following irradiation but also against the additional increase due to heat treatment of irradiated albumin. Thus it will be observed that the irradiated, heat-treated preparation with caprylate practically coincides with the control; without caprylate the curve is even steeper than after irradiation, indicating an additional increase in viscosity due to heat. The estimated intrinsic viscosities⁷ characterizing the six groups of curves in Fig. 1, reading upwards are: 10, 10, 20, 20, 27, and 33, respectively.

Absorption Spectrum Studies

The increased absorption of irradiated solutions of albumin has been reported by others, but it was investigated here, first, to obtain correlations with the changes in viscosity under the conditions of the experiments and, second, to determine whether increases in absorption could be prevented or minimized by caprylate, in view of the stabilizing action caprylate exerts against viscosity changes.

Figure 2 presents the ultraviolet absorption curves for aliquots of the same treated solutions which yielded the viscosity results shown in Fig. 1. It will be observed that: (a) heat alone does not alter the absorption spectrum; (b) irradiation alone increases the absorption; (c) heating after irradiation intensifies the absorption over the entire spectral region examined; (d) addition of sodium caprylate, which has a negligible effect on absorption of untreated albumin, has resulted in the maintenance of a lower absorption intensity following irradiation when comparison is made with the albumin solution irradiated without caprylate present (compare curves III and V); and (e) subsequent heating intensifies the absorption of the caprylate-containing irradiated albumin solution in the region of the maximum without increasing the absorption in other regions.⁸

Effect of the Addition of Stabilizers After Irradiation

The salts of aromatic acids were not added to albumin solutions before irradiation since it was realized that their efficient absorption of

⁷ The intrinsic viscosity $[\eta]$ is the value for the function $100(\eta_r - 1)/c$ extrapolated to $c = 0$. η_r represents the viscosity relative to the solvent and c represents the albumin concentration in g./100 ml. of solution.

⁸ Absorption spectra were determined at pH 6.0 as well as at 7.4. The changes are less striking at the lower pH but in the same order and direction as at pH 7.4.

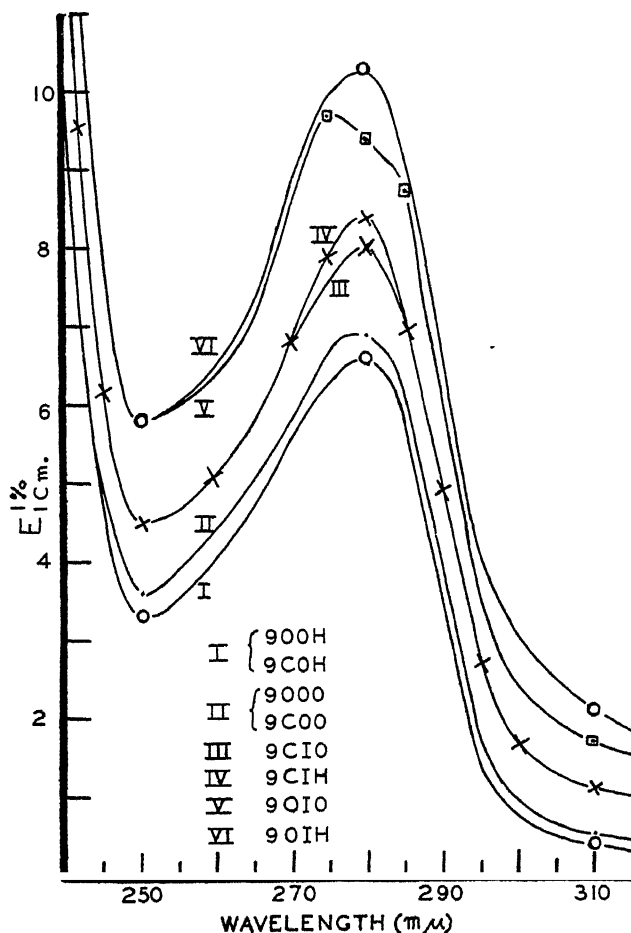


FIG. 2. The influence of sodium caprylate upon the ultraviolet absorption of irradiated and heated albumin solutions. The absorption spectra have been determined for aliquots of the treated albumin samples which provided the viscometric data presented in Fig. 1. Extinction coefficients were determined for 0.1% albumin solutions adjusted to pH 7.4 before measurement. The legend identifies the various treatments and the pH at the beginning of treatment. The code of the legend is explained in the caption of Fig. 1. The intervals at which optical density readings were taken on all curves are as shown in curve IV, even though only a few of the points are presented on the other curves.

ultraviolet light and their probable breakdown would further complicate investigation. However, the influence of acetyltryptophan or caprylate upon changes occurring after irradiation has been investigated. The results are presented in Table I. We have shortened the irradiation

TABLE I

The Effect of Caprylate or Acetyltryptophan Concentration upon the Viscosity of Bovine Albumin Following Irradiation and Heating

| Irradiation time at 82 mμ. (20% albumin) | Heating conditions (18% albumin) | | | | Albumin, 3%, in 0.15 M NaCl | |
|---|----------------------------------|------------------|-------------------------------|----------------------------|---|---------------------|
| | Time | Temper- ature | Sodium caprylate ^a | | Viscosity relative to solvent at 30 | Gross appearance |
| | | | Concen- tration | Mole ratio ^b | | |
| hr. | hr. | °C. | M | | | |
| 0.5 | 0.75 | 61.5 | 0 | — | 1.45 | Cloudy |
| 0.5 | 0.75 | 61.5 | 0.023 | 8:1 | 1.16 | Clear |
| 0.5 | 0.75 | 61.5 | 0.092 | 34:1 | 1.16 | Clear |
| 1.5 | 0.75 | 61.5 | 0 | — | gel ^c | Cloudy |
| 1.5 | 0.75 | 61.5 | 0.023 | 8:1 | 1.16 | Clear |
| 1.5 | 0.75 | 61.5 | 0.092 | 34:1 | 1.17 | Clear |
| 1.0 | 0.50 | 57.0 | 0 | — | 1.30 | Cloudy |
| 1.0 | 0.50 | 57.0 | 0.0018 | 1:2 | 1.26 | Cloudy |
| 1.0 | 0.50 | 57.0 | 0.0045 | 3:2 | 1.20 | Cloudy |
| 1.0 | 0.50 | 57.0 | 0.0090 | 3:1 | 1.18 | Clear |
| Sodium acetyltryptophan | | | | | | |
| | | | M | Mole ratio | | |
| 1.0 | 0.50 | 60.0 | 0 | — | 1.40 | Cloudy |
| 1.0 | 0.50 | 60.0 | 0.001 | 1:3 | 1.27 | Cloudy |
| 1.0 | 0.50 | 60.0 | 0.003 | 1:1 | 1.21 | Cloudy |
| 1.0 | 0.50 | 60.0 | 0.012 | 4:1 | 1.16 | Slightly cloudy |
| 1.0 | 0.50 | 60.0 | 0.030 | 11:1 | 1.16 | Clear |

^a Sodium caprylate or sodium acetyltryptophan were added following irradiation but prior to heating.

^b Mole ratio (compound albumin) is calculated using the g./l. concentration and an assumed mol. wt. of 70,000 for the bovine albumin.

^c The gel is 18% albumin, so dilution to 3% albumin for a viscosity test was impossible.

time and adjusted the heating conditions so as to produce viscosity increases in the solutions lacking stabilizers.

In the absence of stabilizer and using heating conditions as indicated in the table, 0.5 hr. irradiation of 20% albumin is insufficient to produce a gel, while 1.5 hr. irradiation resulted in gel formation. Considering the data within each experiment of the four recorded in the table, it is apparent that extremely small concentrations of sodium caprylate or sodium acetyltryptophan are effective in reducing the viscosity developed on heating the irradiated solutions of albumin. There is little apparent difference in the efficacy of sodium acetyltryptophan as compared to sodium caprylate, since the mole ratios required to prevent a viscosity increase during heating are about the same. The relative viscosities given are of qualitative significance only. It is obvious that the formation of aggregates during heating at these higher temperatures is leading to more structural viscosity and greater turbidity in the absence of stabilizer or when insufficient stabilizer is present.

DISCUSSION

It would be interesting to know how caprylate and other compounds similar in action are able to "stabilize" bovine albumin against changes in viscosity and other properties effected by the denaturing action of ultraviolet radiations. Before attempting such an explanation it is necessary to summarize briefly previous observations concerning the mechanism of "denaturation" of protein by ultraviolet radiations.

Many of the physical and chemical changes which follow irradiation of proteins with ultraviolet radiations are summarized in a recent review by McLaren (7). Clark (6) has presented evidence for the occurrence of a three-step denaturation following irradiation of egg albumin: (a) a photochemical reaction, independent of temperature; (b) a thermochemical reaction resulting in the formation of light scattering material, and (c) the conversion of the turbid aggregates into an actual precipitate. Mirsky and Pauling (8) suggested that the primary effect of irradiation of proteins with ultraviolet light is the breaking of the C-N bonds in the vicinity of aromatic residues of peptide chains, for which experimental evidence is found both in studies of surface films (9-13) and of solutions (14,15). Following such photolysis these authors assume that the hydrogen bonds between peptide chains are more easily broken down by thermal agitation, leading to various secondary reactions.

The way in which ultraviolet treatment as reported here increases the viscosity over and above that obtained by denaturation in DSS might be explained by assuming: (a) first a peptide-bond breakage, then unfolding, and finally aggregation (the aggregates not separating under the subsequent DSS treatment); (b) greater freedom of unfolding, either by whole molecular chains or fragmented chains, subsequent to additional treatment (DSS alone, ultraviolet and DSS, ultraviolet and heat and DSS); or (c) increasing freedom of unfolding, on the part of molecular chains, without appreciable photolysis. The data given in this paper do not enable one to choose between alternatives (a) and (b), but alternative (c) is probably eliminated, since the changes in absorption spectra could scarcely be obtained without the occurrence of a photochemical reaction. Alternative (a) is supported by the recent finding by Roberts that free-radical polymerization occurs in irradiated protein solutions as indicated by polymerization of acrylonitrile which is not so affected by ultraviolet light alone; serum albumin solutions showed increased aggregation when irradiated in nitrogen (as measured by osmotic pressure) and a decrease in aggregation, after a preliminary increase, when exposed in oxygen; other data gathered in this study are in agreement with postulation of free-radical formation (16).

The mechanism by which caprylate "stabilizes" against such increases in viscosity produced by ultraviolet light might be (a) by preventing peptide bond breakage, (b) by preventing the unfolding of whole or fragmented peptide chains, or (c) by preventing the aggregation of chains. The first possibility seems very unlikely since the only way one could prevent a photolytic reaction would be by the induction of a reaction leading to a different product. On the basis of available evidence it is not possible to choose between the other two possibilities. The fact that "stabilizers" added *after* irradiation are effective (Table I) indicates that caprylate probably interferes with the completion of the secondary or thermosensitive reactions occurring in irradiated albumin solutions.

As to the mechanism by which caprylate and similar compounds "stabilize" against the increase in absorption which follows irradiation with ultraviolet light, little can be said because little is yet known concerning the mechanism by which such absorption changes occur in the absence of caprylate. A mere splitting of the protein, assuming the breakage of some peptide bonds, is not likely to increase the absorption of the resulting protein-amino acid mixture. The last statement follows

from the observation that the ultraviolet absorption spectrum of a pure protein is an approximate summation of the spectra of the aromatic amino acids contained in its molecule. Secondary reactions are probably responsible for the changes in absorption which occur. Some alternative reactions to be considered are the following: (a) certain groups in the protein form more strongly absorbing chromophores; (b) reactions between protein molecules lead to the formation of such chromophores; and (c) such chromophores are formed from the amino acids or other fragments split out of the protein. The second alternative, if substantiated, could provide a simultaneous explanation for the increase in absorption intensity and viscosity reported in this paper. Evidence favoring the third alternative is provided by the increased ultraviolet absorption of solutions of irradiated peptides (15). Present evidence is not conclusive in ruling out either the second or third alternative.

SUMMARY

1. A method of investigation of ultraviolet-irradiated albumin solutions has been developed. This consists of denaturation with dodecyl sodium sulfate of the previously irradiated albumin; this acts as an "amplifier" of the effects of irradiation or other treatment.

2. Using this method it has been demonstrated that the viscosity of albumin is increased by irradiation.

3. The viscosity of irradiated albumin is further increased by subsequent heat treatment for 1 hr. at 50°C., a treatment which has no effect on controls.

4. Such viscosity increases may be prevented by the use of caprylate.

5. The ultraviolet absorption of bovine albumin is intensified over the wavelength region examined following dosages of ultraviolet radiation which produce viscosity changes. The ultraviolet absorption of irradiated albumin solutions is further increased by subsequent heating at 50°C. Caprylate is able to prevent or minimize such increases in absorption during these treatments.

6. Hydrogen-ion concentration affects both viscosity development by subsequent denaturation in irradiated solutions as well as their absorption of ultraviolet light; but only a small pH range was studied.

7. The literature is briefly reviewed and attempts are made to explain the various results enumerated above in terms of previous findings.

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Reactivation of Dispersed Chloroplast Material by Reaggregation¹

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INTRODUCTION

The discovery that isolated chloroplasts can carry on the photochemical evolution of oxygen from water permits the study of one reaction of photosynthesis in a cell-free medium. Seemingly this puts us one step nearer to the possibility of separating photochemically active and inactive components of chloroplasts. The next step toward isolation and identification of a possible photosynthetic enzyme would require subdivision of the substance of the chloroplast to colloidal particles.

In order to see how far subdivision of chloroplast material could be carried without losing its photochemical activity, and in order to reduce the particle size to a point which might make possible the separation of an enzyme, colloidal dispersions (1) of chloroplast material were prepared. Half or more of the original activity of chloroplasts is lost in the process of dispersion but a usable fraction of the remaining activity can be preserved for several days (2).

Some of the procedures ordinarily used for protein fractionation were applied to dispersed chloroplast material in hope of concentrating an active fraction. No such concentration was obtained, but unexpected activation and inactivation effects on the material were observed. These effects are described in this paper.

EXPERIMENTAL PROCEDURES

Chloroplasts were isolated from Swiss chard (*Beta vulgaris* var. *cicla*). Preparation of colloidal dispersions of chloroplast material and determination of their activity have been described (1,2). The activity of chloroplasts and dispersions were both measured

¹ This work was supported in part by a grant from the Research Corporation.

at the time the dispersion was made. Initial $Q\%_b$ may be calculated by multiplying activity by 13.44 (2). Dispersions in water were kept at 0°C ., those in 15% methanol at -5° , in the dark. The experimental work was done in a cold room at 1° .

Attempts to fractionate dispersions in water by precipitation with neutral salts such as $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 , or NaCl showed that salt concentrations of 0.5–1 M were needed to start precipitation and that the precipitated material was partially or wholly inactivated. Precipitation with alcohol occurred slowly and only at high concentrations, yielding an inactive precipitate.

When a low concentration of salt was added to a dispersion in dilute methanol, an increase in activity resulted. As the activity of the dispersion is much lower than that of the chloroplasts, we may regard the effect of salt and methanol as a partial restoration of the activity lost by dispersion.

RESULTS

Activation With KCl

Portions of a dispersion in water were adjusted by the addition of water, aqueous methanol, and KCl solution so that each dilution had the same concentration of chloroplast material, the methanol concentration shown at the right in Fig. 1, and the KCl concentration indicated by the abscissas. Activities are plotted as per cent of the activity

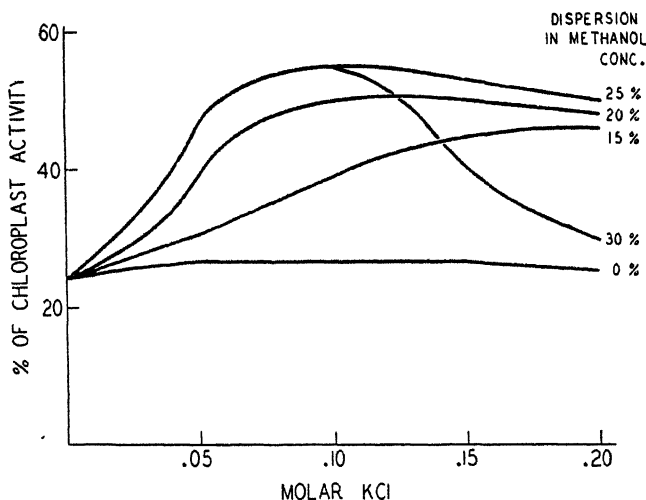


FIG. 1. Reactivation of dispersion by KCl and methanol. Aliquots of one dispersion were brought to indicated methanol concentration before addition of KCl . Activity measured after addition of KCl .

of the chloroplasts before dispersion. In this experiment chloroplast and dispersion activity were 65 and 16, respectively. The curves in Fig. 1 show the effect of different KCl and methanol concentrations on the degree of reactivation produced. As a routine test for the reactivation effect, 0.1 *M* KCl and 20% methanol were used.

The degree of reactivation is reproducible with a given dispersion at a given time, but varies with different preparations and with the age of a single preparation. The reactivation effect is lost sooner on storage than is the activity. Reactivated dispersions in some instances have nearly reached the activity of the chloroplasts before dispersion.

The full activation effect is attained quickly after addition of KCl, remains constant about 1 hr., then is lost slowly and disappears in about 24 hr. Measurements were made 15 min. after addition of KCl.

The degree of reactivation decreases with dilution of the chloroplast material. At 0.2 mg./ml. of chlorophyll the reactivation was twice that observed at 0.02 mg./ml.

In each case of an appreciable reactivation effect the mixtures became turbid. Mild centrifugation caused sedimentation of the precipitated material. The precipitate, resuspended in fresh 20% methanol-0.1 *M* KCl solution, had the same activity as the mixture from which it came. In 20% methanol without KCl, the precipitate partially redispersed with some lowering of its activity. Thus it seems clear that reactivation and precipitation occur together. We have observed no case of reactivation without precipitation.

Activation by Other Salts of Monovalent Cations

Is the reactivation a specific effect of KCl, of K ion or Cl ion? Using a dispersion in 20% methanol and a salt concentration 0.05 *M*, several salts were tested. The results are shown in Fig. 2. The activity of the untreated dispersion was 17.

The effects of NH_4F and KNO_3 are hardly significant. The potassium phosphate used was a mixture of primary and secondary, pH 6.5. KBr , NaCl , and NH_4Cl each show about two-thirds the effect of KCl. Sulfates, with twice the cation concentration per mole, show greater activation than the corresponding chlorides. The reactivation effect is not caused specifically by K ion or Cl ion, but by a number of neutral salts. In order of increasing effectiveness the cations are Li^+ , Na^+ , NH_4^+ , and K^+ ; the anions are F^- , NO_3^- , PO_4^{---} , Br^- , and Cl^- or SO_4^{--} .

In contrast to the effect of KCl, which remains almost unchanged for 2 hr., the greater initial activation by $(\text{NH}_4)_2\text{SO}_4$ disappears rapidly and in 2 hr. changes to a 70% inactivation.

Activation by pH Adjustment

Aliquots of a dispersion in 20% methanol were adjusted to pH 4-9 in half-unit steps, and at a 0.05 *M* potassium phosphate concentration. The activation at a pH of 6.5 duplicated that shown in Fig. 2. At a pH greater than 7 the activation was smaller or absent. On the acid side of pH 6.5 the activation increased, reaching a maximum of 215% at pH 5. At pH's of 4.5 and 4, precipitation and total inactivation occurred.

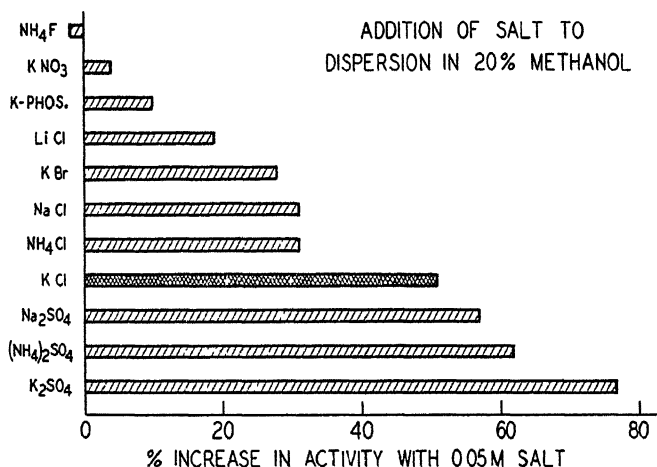


FIG. 2. Reactivating effect of various salts. Aliquots of a dispersion in 20% methanol were made 0.05 *M* with respect to indicated salt. Increase in activity after adding salt is shown by length of bars.

In order to separate the pH effect and the salt effect, the same range of pH was attained by adding small volumes of 0.0001 *N* HCl to the dispersion. The degree of activation was roughly equal to that obtained at corresponding pH values in the 0.05 *M* phosphate series.

Effect of Divalent Metals

Salts of divalent metals are much more effective precipitants of dispersed chloroplast material, and produce activation effects at much

lower concentrations, than salts of monovalent metals. Dispersions in 15% methanol are preferable to those in 20% when testing the effect of divalent metals.

It was thought that the effect of divalent metals might be counteracted by the use of versene [ethylenedinitrilotetraacetic acid]. The acid versene was partially neutralized to pH 6.5 with KOH. Duplicate samples of dispersion in 15% methanol were treated with salt solution, also in 15% methanol, to give 0.0005 *M* salt and were allowed to stand 5 min. To one sample, versene in 15% methanol was added to make 0.015 *M*, and to the other sample an equal volume of 15% methanol was added. The resultant activities are shown in Fig. 3.

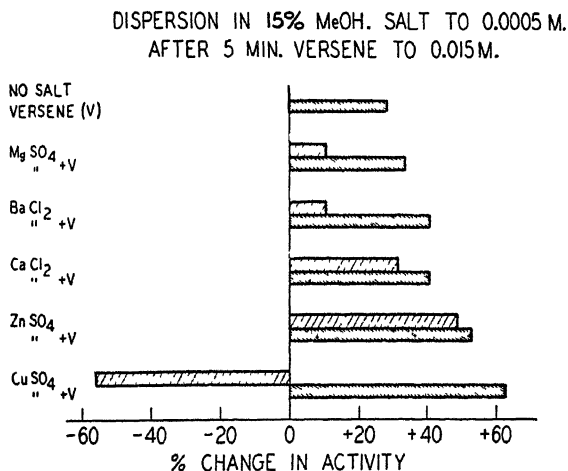


FIG. 3. Effect of salts of divalent metals and of versene on dispersion in 15% methanol. See text for experimental details.

The activities are plotted as per cent change from 19, the activity of the untreated dispersion, indicated as 0. The first pair of samples were blanks, receiving 15% methanol instead of salt solution. The one without versene shows no change in activity due to dilution. The one treated with versene, alone, shows 30% activation, probably a salt effect similar to the ones already described. The activating effects of Mg, Ba, Ca, and Zn were enhanced rather than removed by subsequent treatment with versene. The 55% inactivation by copper, however, is not only restored by versene, but the mixture is reactivated to 63% above the untreated

dispersion. Using 0.003 *M* CuSO₄, the dispersion was completely inactivated and no activity was restored by subsequent versene treatment.

The effects with HgCl₂ and versene were similar to those observed with CuSO₄. At 0.01 *M*, ZnSO₄ had about the same activating effect as 0.1 *M* KCl.

Participation of Lipide in Activation Effect

Next to protein, the most abundant constituent of chloroplasts is lipide, about 25% in our chard chloroplasts. When a dispersion in water is shaken with an equal volume of low-boiling petroleum ether and the two layers are separated, between 2% and 3% of the total lipide content is found in the petroleum ether. The activity of the extracted dispersion is about 0.75 to 0.5 its former value, but the salt activation effect is either much reduced or altogether absent. Restoration of the activity and of the salt activation effect can be brought about merely by shaking the extracted dispersion with a little ethyl ether and removing the ether *in vacuo*.

DISCUSSION

It should be emphasized that we are not dealing here with an activation due to trace amounts of chloride or other ion, but rather with the effect on the colloidal properties of dispersed chloroplast material caused by treatment of it with 15% or 20% methanol and concentrations of neutral salts up to 0.2 *M*. The activations which we have described take place before the measurement of activity is made.

Dispersions and the precipitates obtained from them by salt activation procedures were analyzed for chlorophyll and nitrogen content. From the calculated protein/chlorophyll ratios there was no evidence that fractionation of the material had occurred, other than a separation of the green precipitate from colorless protein remaining in solution. As much as one-third of the original nitrogen content of the dispersion was left in solution after all of the green material was precipitated. Fractional precipitation of the green material by successive small additions of salt to a dispersion in 20% methanol gave precipitates of nearly the same activity and protein/chlorophyll ratio, about 10 to 1 by weight.

Since the salt activation effect is always accompanied by precipitation, it seems probable that the effect depends on an increase in the particle size of the dispersed material. A suspension of whole or lightly fragmented chloroplasts shows little if any increase in activity when subjected to the same salt activation procedure used for dispersions.

The loss of activity through dispersion of chloroplast material and the restoration of activity through reaggregation suggest that the activity is a surface phenomenon. The activation can be caused by a variety of precipitating agents, from which it appears that it is the physical state of the precipitate rather than the identity of the agent which accounts for the activation.

Dr. S. L. Chen, working in this laboratory, tested the effect of streptomycin on dispersed chloroplast material. He found that 0.0004 *M* streptomycin or 0.1 *M* KCl produced almost identical activation of dispersions in 15% methanol. Thus, a quite different agent from the ones previously tested produced the same sort of precipitation-activation effect.

The salt activation effect observed with dispersed chloroplast material is not without parallel. Several workers dealing with respiratory enzymes have noted increased activity in the presence of salts [cf. (3)]. In measuring the oxidative powers of lysed cells, Utter (4) observed a salt activation effect accompanied by precipitation, similar to the effect described here.

Large effects on the activity and on the salt activation of dispersions due to removal of a small part of their lipide content can be explained, or perhaps rationalized, as a surface phenomenon. It may be that shaking an aqueous dispersion with petroleum ether washes some surface lipide from the particles and changes their photochemical properties. Subsequent treatment with ethyl ether as described may transfer fresh lipide from the interior of the particles to the surface, thereby restoring their activity.

The photochemically active constituent of chloroplasts does not at present appear to be so simple an entity as a chlorophyll-bearing protein. Lipide components other than chlorophyll seem to take part in the photochemical activity of chloroplast material. Aside from the separation of some colorless protein, fractionation of chloroplast material into active and inactive components has not been accomplished. It is hoped that further study of the physical and chemical properties of dispersed chloroplast material will indicate whether the isolation and identification of an active constituent can be accomplished, or whether the entire complex organization of the pigmented substance in chloroplast material is essential to carry out its photochemical activity.

SUMMARY

The photochemical activity of chloroplast material decreases as its particle size becomes smaller. Colloidal dispersions have from one-fourth to one-half the activity of intact chloroplasts. Reaggregation of the dispersed material by a variety of agents in the presence of 15% or 20% methanol reactivates the material. The greatest degree of reactivation was obtained with 0.1–0.2 *M* salts of monovalent cations and with 0.0005–0.01 *M* salts of certain divalent cations.

Inactivating effects of CuSO_4 and HgCl_2 in very low concentrations were overcome by the addition of versene to the mixtures. Not only did versene remove the inactivating effect, it reactivated the mixture to a value above the activity before treatment of the dispersion.

Slight decreases in the lipid content of dispersions caused loss of much of the activity and disappearance of the salt activation effect.

The properties of dispersed chloroplast material indicate that its photochemical activity is a surface phenomenon, and that lipid is an essential component for activity.

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Measurement and Stabilization of Activity of Chloroplast Material ¹

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INTRODUCTION

A primary step in photosynthesis is the photochemical liberation of oxygen from water (1), the hydrogen being used in a reduction reaction. Isolated whole or broken chloroplasts can evolve oxygen from water (2) when they are illuminated in the presence of a suitable hydrogen acceptor. This is now known as the Hill (3) reaction. Since its discovery in 1937, a number of workers have investigated the photochemical activity of chloroplasts, grana, and chloroplast fragments.

Manometric determination of the photochemical evolution of oxygen by chloroplast particles in the presence of an added oxidant has been widely used to measure activity. References to work in this field are given by Arnon and Whatley (4). Warburg (5) used quinone as the oxidant. Holt and French (6) found 100% yields of oxygen by using benzenoneindophenol.

In order to study the activity and other properties of chloroplast material in particles smaller than grana or fragments, we prepared colloidal dispersions (7). In this paper the term "chloroplast material" is used to designate all of the substance of the chloroplasts. In the course of experiments on the properties of dispersed chloroplast material it is desirable to measure quickly the activity of very small samples, and many measurements may be wanted in one experiment. For these reasons the manometric measurement of oxygen evolution seemed unsuitable for our purpose. The progress of the reaction can be followed by other means.

Holt and French (6) described a continuous titration method for

¹ This work was greatly aided by a grant from the Research Corporation.

following the reaction when ferricyanide was used as oxidant. Based on some unpublished experiments of Holt, R. F. Smith, and French, we developed a rapid and accurate photoelectric method for determining activity by measuring the rate of reduction of a hydrogen acceptor.

The first colloidal dispersions we prepared had about a quarter the activity of chloroplasts, and lost that activity in 24–36 hr. Methods were found for increasing the activity of dispersions and for preserving a usable fraction of that activity for several days.

EXPERIMENTAL PROCEDURES

Preparation of Dispersed Chloroplast Material

Chloroplasts were isolated from leaves of *Beta vulgaris* var. *cicla*, commonly called Swiss chard or spinach beet. Leaves, freed of stems and midribs, were frozen overnight at about -5° . This freezing caused some loss in activity of the chloroplasts, but greatly increased the percentage of colloiddally dispersed material obtained from them. The frozen leaves were triturated at 0° in a Waring Blendor with shaved ice and water. Blending was stopped when leaf particles were no longer visible, usually after 1 min. The slurry was filtered by suction through flour sacking in the cold room at 1°C . The filtrate was centrifuged 15 min. at $12,000 \times g$ in a Serval angle-head centrifuge equipped with a bearing cooler and operated in the cold room. The yellow-brown liquid containing only traces of chlorophyll was discarded. The tightly packed sediment of whole and broken chloroplasts was separated with a spatula from the underlying starch and was suspended in enough distilled water to give a chlorophyll content of 2 mg./ml., or about 30 mg./ml. of chloroplast material. This chloroplast suspension was forced through a steel needle valve at 20,000 lb./sq. in. (7). The valved suspension was diluted to 0.5 mg./ml. chlorophyll, then was centrifuged 30 min. at $12,000 \times g$. Not much more chloroplast material than that represented by 0.3–0.4 mg./ml. chlorophyll would remain in dispersion. Dilution of the valved suspension to 0.5 mg./ml. is based on this apparent "solubility" of the dispersed material, and allows for the sedimentation of the undispersed part. The supernatant liquid was separated from the sediment and any sludge, which were discarded.

The term "dispersion" refers only to the supernatant liquid containing chloroplast material in such a state of subdivision that it is not sedimented by 30 min. centrifugation at $12,000 \times g$. Dispersions are dark green, appear free of turbidity when viewed by transmitted light, but show a strong Tyndall effect.

Chlorophyll Determination

From 0.1 to 2.0 ml. of sample is placed in a 6×125 mm. Pyrex test tube. Enough 5% KOH in methanol is added to make 5.0 ml. and mixed well with the sample. The tube is heated 3 min. in a water bath at 63° , then is centrifuged. The clear green solution of saponified chlorophyll is decanted into a colorimeter tube and its transmission measured in a Klett-Summerson photoelectric colorimeter, using red filter No. 66 to minimize interference by yellow pigments. The calibration curve is determined from

chlorophyll solutions standardized by spectrophotometric measurement, using Comar and Zscheile's constants (8). The average time for a chlorophyll determination, where several are run simultaneously, is 5 min. and the results are accurate to $\pm 2\%$.

Measurement of Photochemical Activity

The reaction measured is the reduction of the blue dye, 2,6-dichlorobenzeneindophenol, to its colorless form in the presence of illuminated chloroplast material. The apparatus used is a recording photoelectric colorimeter.

A 200-w. Mazda projection lamp is shielded by a chimney with a 3-cm. opening at the filament level. Light is focused on the reaction cell by 2 lenses. The cells are 1 cm. square, 6 cm. deep, with plane-parallel sides. Corning filters Nos. 3480 and 9780 isolate the spectral band corresponding to the absorption maximum of the dye from the light which has passed through the 1-cm. reaction cell. A piece of paper with *ca.* 5% transmission is placed between the Corning filters and the phototube. Thus it is possible to have a high light intensity in the reaction cell without overloading the phototube.

The voltage output of the R. C. A. No. 926 phototube, amplified by the circuit shown in R. C. A. form PT-29R1, Fig. 13, is fed into a potentiometer consisting of a wire-wound fixed resistance in series with a decade variable resistance box. The voltage drop across the decade box resistance, 10–50 ohms ordinarily, is fed into a Brown Electronic Strip Recorder (potentiometer type), model 153X12V-X-30, range 0–2.5 mv. A Stabiline voltage regulator furnishes a constant voltage A.C. supply. A regulated D.C. power supply for the phototube and its amplifier is fed from the Stabiline regulator. Light intensity is controlled by a variable transformer between the Stabiline regulator and the lamp.

The reaction cell is held on the outside of a watertight housing for the filters and phototube; both are in a water bath thermostatically maintained at 15°.

Using standardized screens it is found that recorder deflections are directly proportional to light intensity. Within the range of concentration used, zero to 0.000033*M*, the transmittance of the dye solution obeys Beer's law. Thus recorder deflections are convertible to dye concentrations.

The photochemical reduction of dye, *within empirically determined limits of concentration*, can be expressed as a first-order reaction by the equation: $k = 1/t \ln a/(a - x)$. The initial and final dye concentrations are *a* and *a - x*, respectively, after *t* sec. illumination. Since *k* has a small numerical value, it is multiplied by 100 to give a more convenient activity coefficient, *K*. *K* divided by the concentration in mg./ml. of chlorophyll in the reaction mixture expresses the activity of the sample of chloroplast material. Calculation of oxygen evolution from activity determined as described gives values in substantial agreement with those reported by Arnon and Whatley (4) for whole and fragmented chloroplasts.

The value $Q_0^{O_2}$ (cu. mm. O_2 /hr./mg. chlorophyll) as used by many workers is calculated from the volume of oxygen evolved in a specific number of minutes early in the course of a reaction. It is assumed that the rate of O_2 evolution is constant, that is, that the measured rate equals the initial rate. This criterion will not be met under the conditions chosen for measurement of activity as described here. It is apparent from the equation for a first-order reaction that the rate of O_2 evolution (dye reduction) will decrease as the reaction proceeds. The initial $Q_0^{O_2}$ can be calculated from the

observed velocity constant of the reaction, and will be proportional to the activity as defined above. Activity $\times 13.44 = \text{initial } Q_0^2$.

For each determination of activity, duplicate samples are put in reaction cells. Water to make 3 ml. is added to one sample, which is the blank. Water is added to the other sample to make 2 ml., followed by 1 ml. of dye solution. The dye solution contains 0.0001 *M* sodium 2,6-dichlorobenzeneindophenol, 0.01 *M* KCl, and 0.04 *M* potassium phosphate, pH 6.5. The sample with dye is kept dark while the apparatus is adjusted by use of the blank. The decade box is adjusted to produce full scale recorder deflection when the light is on. The dark or zero deflection of the recorder is set by means of the phototube amplifier. Both adjustments are rechecked, then the blank is replaced by the sample with dye. With the recorder running, the light is turned on. The change in deflection of the recorder is noted after 120 sec. illumination. From a previously prepared table the corresponding value of *K* is found.

During the 120 sec. of illumination the reaction follows the first-order equation within satisfactorily wide limits of dye concentration. The same value of *K* is found for 40, 80, or 120 sec. if (a) the initial dye concentration is not more than 20% different from 0.000033 *M* in the reaction mixture, and (b) if not more than half the initial dye present is reduced in 120 sec. When the initial dye concentration is outside the limits mentioned, the reaction is still first order but with a different value of *K*. When more than half the dye has been reduced, the reaction no longer follows the first-order equation.

TABLE I
Relation Between Activity and Concentration of Chloroplast Material

| Sample | Chlorophyll in react. mixt. | Recorder deflection | <i>K</i> | Activity, <i>K</i> /chlorophyll |
|------------|-----------------------------|---------------------|----------|---------------------------------|
| <i>ml.</i> | <i>mg./ml.</i> | <i>mv.</i> | | |
| 0.1 | 0.0154 | 0.84 | 0.216 | 14.0 |
| 0.2 | 0.0309 | 1.03 | 0.447 | 14.5 |
| 0.3 | 0.0463 | 1.18 | 0.643 | 13.9 |
| 0.4 | 0.0618 | 1.34 | 0.876 | 14.2 |
| mean | | | | 14.2 ± 0.3 |
| 0.5 | 0.0772 | 1.43 | 1.026 | 13.3 |
| 0.6 | 0.0926 | 1.43 | 1.026 | 11.1 |

The value of *K* is directly proportional to the chlorophyll concentration of the mixture, unless the latter is great enough to reduce the light intensity in the reaction cell below the saturation level. Table I shows the proportionality of *K* and mg./ml. of chlorophyll for different amounts of the same dispersion. From 0.1 to 0.4 ml. of sample the activity was 14.2 ± 0.3 . The 0.5-ml. sample shows a lower activity because of too great light absorption in the reaction cell. Increasing the sample from 0.5 to 0.6 ml. caused no increase in the dye reduced. The lower limit of sample size is determined by the activity of the sample, the upper limit by its chlorophyll content.

Including all manipulations, an activity measurement takes only 5 min. The quantities of material needed are very small, as shown in Table I. The amount of dye

reduced in a typical measurement corresponds to the evolution of about 0.4 cu. mm. of oxygen. Measurements are reproducible within 5% in most cases, within 2% for samples having good activity.

RESULTS AND DISCUSSION

An indication of the particle size in our dispersions was obtained by recentrifuging them at $12,000 \times g$ in the Serval. About one-fourth of the material sedimented in 30 min., about three-fourths in 5 hr. An ultracentrifuge test performed by Mr. Robert Lashbrook through the courtesy of Dr. H. S. Loring indicated a heterogeneous distribution of particle size. A mean "molecular" weight of 6-7 million was calculated for 90% of the material, 10% having a smaller size. A run in a Spinco analytical centrifuge kindly made for us by Drs. Clark Griffin and Lafayette Noda, showed no boundaries. About one-fourth of the material sedimented in 15 min. at $20,000 \times g$ and most of the remainder in 10 min. more at $60,000 \times g$.

Electron microscope photographs of another dispersion, taken by Mr. Ernest Fullam of the General Electric Co., showed a mixture of particles with most smaller than 20 A., some of 80 A., and a few 250-A. particles containing the 80-A. units.

Loss of the photochemical activity of dispersions is rapid at high temperature or under bright light. It is necessary to keep the preparations cold, and to expose them only to light of low intensity while working with them. Freezing of dispersions often causes partial inactivation. Loss of activity is rapid at room temperature, and a few minutes at 35° or higher causes complete inactivation. When all of the preparative operations are performed at 0° and the dispersion is stored at 0° in the dark, half the initial activity is lost in 24-36 hr.

Many agents were tried for stabilizing the activity of dispersions. Potassium chloride was reported to have an activating effect on washed broken chloroplasts (5). Neither KCl nor dilute phosphate buffers had an activating or stabilizing effect on dispersions. Buffered dispersions were slightly less active than unbuffered ones, and lost activity more rapidly. A number of workers have used 0.5 *M* sucrose as the medium for handling chloroplasts. Sucrose and also propylene glycol have a small stabilizing effect on the activity of dispersions.

In methanol concentrations of 40% and lower, dispersions lost activity more slowly than those in water. The stabilizing effect of different concentrations of methanol on aliquots of one dispersion is shown in

Fig. 1. Each aliquot was diluted with the same volume of water or of aqueous methanol so that the chlorophyll content was the same in each, and the methanol concentrations those indicated in the figure. Samples were kept in the dark at 1°. The heavy line shows the loss of activity without methanol. There is no significant difference between the effect of 15, 20, or 25% methanol. The effects of 30% and 10% methanol are approximately equal, also those of 5% and 40% methanol.

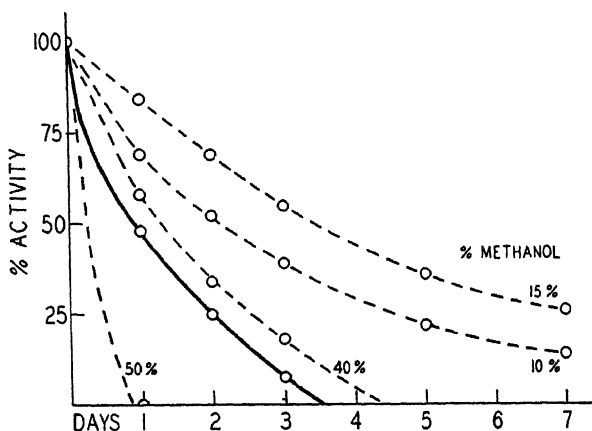


Fig. 1. Stability of the same dispersion in different methanol concentrations, at 1° in the dark. Sample without methanol is indicated by solid line.

The dispersion used for obtaining the data in Fig. 1 had an activity of 20 when prepared; the chloroplasts at time of dispersion had an activity of 80. The data are plotted as per cent of the activity of the dispersion when prepared, instead of the measured activities. This is a matter of convenience because the percentage change in activity of dispersions under given conditions is fairly reproducible, although the numerical value of the activity varies from one preparation to another. Many stability curves similar to Fig. 1 were determined. In each case the loss of activity corresponded roughly to a reaction of zero order, the rate being determined by the experimental conditions. This shape of the loss of activity curve suggests the use of the half life of activity as an index of stability under different conditions. From Fig. 1 the half life of activity with 0, 10, and 15% methanol is about 1, 2, and 3.5 days respectively. The presence of 15% methanol permits storage of dispersions at

-5° without freezing. Under these conditions, the half life of dispersions is 10 days or more.

The stability of dispersions in 15% methanol is less at lower concentrations of chloroplast material. Dilutions of one dispersion, activity 15.5 when prepared, were kept at -5° in 15% methanol. Chlorophyll concentration was 0.03, 0.06, 0.09 and 0.24 mg./ml. The half life of activity was 2, 5, 7, and 13 days, respectively.

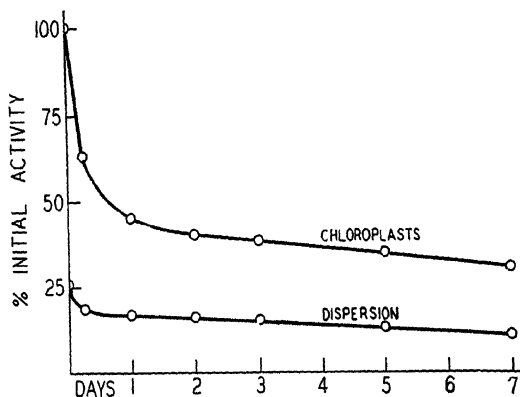


FIG. 2. Stability of chloroplasts before and after dispersion. Both samples in 15% methanol at 1° .

Ethanol was tested for stabilizing effect in 21.5% concentration, equimolar with 15% methanol. The two did not differ significantly at -5° . At 1° , ethanol was a little less effective than methanol. At 8° ethanol was about half as good as methanol; at 18° , ethanol did not show a significant stabilization, whereas methanol was still effective.

The rapid loss of activity of chloroplasts immediately after isolation is slowed but little by 15% methanol. When the activity of the chloroplasts has fallen to about 40% of its initial value, 15% methanol slows the further loss to about the same rate as in the case of dispersions. Figure 2 shows the loss of activity of a chloroplast suspension and a dispersion made from it, both in 15% methanol at 1° . The activity of the chloroplasts was 87 at the time of dispersion, the activity of the dispersion was 22. In Fig. 2 the chloroplast activity is plotted as 100%, and the time of dispersion as zero time.

In order to obtain chloroplasts of the highest activity it is necessary to use a small amount of leaves and to carry out the isolation quickly. For example, a small quantity of chloroplasts was isolated in a few minutes at 0° and the suspension in water was kept at 0°. The activity was 207 when first measured. In 20 min. the activity was 76% of the initial value, in 40 min. 52%, and in 1 hr. only 39%.

Full advantage of the stabilizing effect of 15% methanol can be taken by using it for trituration of the leaves in the Waring Blendor, and by carrying out the following steps in the presence of 15% methanol. In this way, the entire preparation may be done at -5°. Dispersions prepared in water usually have about one-fourth the activity of the chloroplasts. When the whole procedure is carried out in 15% methanol the dispersion has between one-third and one-half the activity of the chloroplasts.

SUMMARY

A recording photoelectric colorimeter is described for measuring the rate of photochemical reduction of 2,6-dichlorobenzenoneindophenol in the presence of illuminated chloroplast material. The rate of dye reduction divided by the chlorophyll content of the sample gives a measure of the photochemical activity. A dye-reduction measurement can be made in 5 min. and requires very little material. In connection with a rapid method for chlorophyll determination, many activity measurements can be made in a day.

The preparation of colloidal dispersions of chloroplast material is described and some of the properties of the dispersions are discussed. Dispersions prepared in water lose their activity in a day. By use of 15% methanol as the medium, the half life of activity of dispersions is extended to 10 days or longer.

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The Antimicrobial Activity of Several Substituted Pyrones

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INTRODUCTION

Studies on the correlation of chemical structure with bactericidal activity have shown that many compounds containing an *alpha*, *beta*-unsaturated ketone linkage have antimicrobial properties (1,2,3,4). This correlation may be related in part to the reaction of the unsaturated ketone with thiol groups of enzymes essential in bacterial metabolism. The literature on this subject has been reviewed by Roblin (5). Since 1,2- and 1,4-pyrone derivatives contain the *alpha*, *beta*-unsaturated ketone structure, a series of these compounds has been prepared and tested for antimicrobial activity.

MATERIALS AND METHODS

Table I contains a summary of the chemical analyses and the melting or boiling points of the test compounds.

Antimicrobial Activity

Salmonella typhosa and *Micrococcus pyogenes* var. *aureus* were used as representative of pathogenic bacteria belonging to two separate classes as determined by physiological properties. The microorganisms, *Aerobacter aerogenes*, *Saccharomyces cerevisiae*, *Penicillium digitatum*, and *Rhizopus nigricans*, were chosen to represent resistant bacteria, yeasts, and fungi commonly associated with food spoilage.

Culture Media

Brain heart infusion broth (Difco) for *S. typhosa*, *M. pyogenes* var. *aureus*, and *A. aerogenes*.

Malt yeast broth (Difco) for *S. cerevisiae*.

Malt yeast agar (Difco) for *P. digitatum* and *R. nigricans*.

Some of the pyrones tested have limited solubility in water. Therefore, it was necessary to use alcohol or acetone in order to prepare stock solutions of several of the

test compounds. The limiting bacteriostatic concentrations of these solvents were determined and their subsequent use was kept below these limits in the final test solution of each pyrone.

When sodium hydroxide was used as a solvent, pH determinations were made on uninoculated duplicate samples to be certain that the test solutions were in the range of pH 6-8 in the bacterial tests and pH 4-7 in the cases of the yeast and fungi.

TABLE I
Analytical Data, Substituted Pyrones

| Compound | Compound no. | Theoretical (%) | | | Obtained (%) | | | M. P., C.° (°) |
|---|--------------|-----------------|------|------|--------------|------|------|----------------------------|
| | | C | H | N | C | H | N | |
| 1,2-Pyrone, 5-carboxy-(coumalic acid) | 1 | 51.42 | 2.86 | | 51.52 | 3.26 | | 203-206 (Dec.) |
| 1,4-Pyrone, 5-Hydroxy-2-(hydroxymethyl)-(kojic acid) | 2 | 50.66 | 4.29 | | 50.81 | 4.35 | | 152-154 |
| 5-Hydroxy-2-(chloromethyl)- ^c | 3 | 44.86 | 3.12 | | 44.87 | 3.40 | | 163.5-165.0 |
| 2,6-Dimethyl- | 4 | 67.70 | 6.53 | | 67.58 | 6.56 | | 131.5-134 |
| 3-Hydroxy-2-methyl-, (maltol) | 5 | 57.14 | 4.76 | | 57.05 | 4.91 | | 161-164 |
| 3-Hydroxy-2-methyl-, hydroxylated | 6 | 54.50 | 9.16 | | 54.70 | 9.24 | | 245/atm. ^b |
| 6-Carbobutoxy-2,3-dihydro-2,2-dimethyl- | 7 | 63.70 | 8.01 | | 63.69 | 8.34 | | 174-179/20 mm ^b |
| 3-(2,4-Dinitrophenoxy)-2-methyl- | 8 | | | 9.59 | | | 9.49 | 192.0-192.5 |
| 5-(2,4-Dinitrophenoxy)-2-hydroxymethyl- | 9 | | | 9.09 | | | 8.68 | 172-173 |
| 3-Benzoxo-2-methyl- | 10 | 67.76 | 4.43 | | 67.60 | 4.41 | | 111-113 |
| 1,2H-Pyran-2,4 (3H) dione, 3-Acetyl-6-methyl-, (dehydroacetic acid) | 11 | 57.11 | 4.82 | | 57.77 | 4.95 | | 109-111 |
| 3-Benzoyl-6-phenyl- | 12 | 73.97 | 4.11 | | 74.05 | 4.31 | | 171-172 |
| 3-(1-Aminoethylidene)-6-methyl- | 13 | | | 8.38 | | | 8.08 | 207.5-208.5 |
| 3-(1-Anilinoethylidene)-6-methyl- | 14 | | | 5.76 | | | 5.32 | 117.5-120.0 |
| 3-[1-(p-Sulfamyl)anilino]-ethylidene-6-methyl- | 15 | | | 8.69 | | | 8.73 | 251.0-252.5 |
| 3-Acetyl-5-carboxy-6-methyl- | 16 | 50.92 | 3.80 | | 51.38 | 4.04 | | 152-154 |

^a All melting points uncorrected.

^b Boiling point.

^c Chlorine, theoretical 22.12, obtained 21.91.

Source of compounds:

1, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14, 15, 16—The Dow Chemical Co.

2—U. S. Department of Agriculture. Recrystallized.

7—U. S. Industrial Chemicals Inc.

12—Eastman Kodak.

In all cases, double-strength media were prepared, thus allowing for the necessary addition of pyrone solution together with water to give a final adjusted volume. When an agar medium was used the compound was added to the melted agar medium and the mixture was allowed to cool as a slant.

0.1-ml. quantities of a 24-hr. culture of the bacteria and yeast were used in inoculums. *P. digitatum* and *R. nigricans*, the two fungi studied, were grown on broth containing glass beads. After shaking the well-sporulated culture vigorously, an inoculum of 0.1 ml. was streaked onto the surface of a slanted agar medium.

The bacteria *S. typhosa*, *M. pyogenes* var. *aureus*, and *A. aerogenes* were cultured at 37°. The test solutions were checked for growth at 1, 2, and 7 days. Those tubes that were negative after 7 days were subcultured to differentiate between bacteriostatic and bactericidal activity.

S. cerevisiae, *P. digitatum*, and *R. nigricans* were grown at 30°C. Observations for the growth of *S. cerevisiae* were the same as for bacteria. The two fungi were observed after 2, 7, and 14 days. Inhibitory concentrations at 14 days were not checked to differentiate between fungistatic or fungicidal activity.

RESULTS

The results of the antimicrobial tests and the solvent used in each case are summarized in Table II.

DISCUSSION

In all cases the compounds studied inhibited the test organisms at much lower concentrations when the test period was shortened from 7 days to 2 days or less. However, the longer test period was used since we were primarily interested in the behavior of these compounds under practical conditions of use.

Morton *et al.* (6) have reported on the antibacterial activity of kojic acid against a wide number of organisms. In general, they obtained inhibition in a range of 0.05–0.1% against most bacteria. This value is somewhat less than that obtained in this study.

Brodersen and Kjaer (7) have reported on the activity of dehydroacetic acid in a study of certain unsaturated lactones. They report it as being inhibitory at a concentration of less than 1% against both *M. pyogenes* var. *aureus* and *S. typhosa* two of the organisms used in this study.

The differences in values obtained by these laboratories and those reported in this study are not great; therefore, it is quite possible that the variations are attributable to differences in laboratory techniques.

Dehydroacetic acid has a wide antimicrobial spectrum including fungi, yeasts, and gram-positive and gram-negative bacteria. It is unique in that pastes containing as much as 65% of this compound do not produce primary skin irritation nor skin sensitization upon topical application to human subjects (8). Laboratory tests show that dehydroacetic acid is tasteless and odorless at concentrations required to control undesirable microbial growth in certain foods. Furthermore, extensive toxicological studies conducted on rats, dogs, monkeys, and man indi-

TABLE II
Antimicrobial Activity of Substituted Pyrones
Test period—Yeast and bacteria, 7 days; fungi, 14 days

| Compound | Test organism | | | | | | Solvent used in test |
|--|--|--|--|--|--|---|---|
| | <i>Aerobacter aerogenes</i> | <i>Micrococcus pyogenes</i> var. <i>aureus</i> | <i>Salmonella typhosa</i> | <i>Penicillium digitatum</i> | <i>Rhizopus nigricans</i> | <i>Saccharomyces cerevisiae</i> | |
| 1,2-Pyrene, 5-carboxyl-, (coumalic acid) | 0.90-1.20 ^a | 0.75 | 0.60 | 0.60-0.75 | 0.60-0.75 | 0.30 | NaOH |
| 1,4-Pyrene, 5-Hydroxy-2-(hydroxymethyl)-, (kojic acid) | | | | | | | |
| 5-Hydroxy-2-(chloromethyl)-, 2,6-Dimethyl-, 3-Hydroxy-2-methyl-, (maltol) | 0.30-0.40 0.07-0.09 1.50 | 0.50-0.60 0.03-0.05 1.50 | 0.10-0.20 0.03-0.05 1.20-1.50 | 1.50 0.02-0.03 1.20-1.50 | 1.50 0.03-0.04 1.50 | 1.50 0.05-0.07 1.50 | Water Acetone Water |
| 3-Hydroxy-2-methyl-, hydrogenated | 0.75-0.90 | 1.20-1.50 | 0.50-0.60 | 0.10-0.20 | 0.20-0.30 | 0.30-0.40 | Water |
| 6-Carbobutoxy-2,3-dihydro-2,2-dimethyl-, 3-(2,4-Dinitrophenoxy)-2-methyl-, 5-(2,4-Dinitrophenoxy)-2-hydroxymethyl-, 3-Benzoxo-2-methyl-, | 0.40-0.50 0.30 0.06 0.03 0.30-0.40 | 0.60-0.75 0.30 0.06 0.03 0.40-0.50 | 0.30-0.40 0.30 0.06 0.03 0.10-0.20 | 0.40-0.50 0.30 0.06 0.03 0.10-0.20 | 0.75-0.90 0.30 0.06 0.03 0.20-0.30 | 0.60-0.75 0.30 0.06 0.03 0.30 | Alcohol Alcohol Acetone Acetone Acetone |
| 1,2H-Pyran-2,4 (3H) dione, 3-Acetyl-6-methyl-, (dehydroacetic acid) | | | | | | | |
| 3-Benzoyl-6-phenyl-, 3-(1-Aminoethylidene)-6-methyl-, 3-(1-Antiinoethylidene)-6-methyl-, 3-[1-(p-Sulfamylamino)-ethylidene]-6-methyl-, | 0.20-0.30 1.20 0.20-0.30 0.15 | 0.20-0.30 0.20-0.30 0.30-0.40 0.15 | 0.10-0.20 0.20-0.30 0.20-0.30 0.15 | 0.02-0.03 0.20 0.20-0.30 0.02-0.03 | 0.03-0.04 0.20 0.10-0.20 0.02-0.03 | 0.05-0.10 0.20 0.20-0.30 — | Alcohol NaOH Alcohol Alcohol |
| 3-Acetyl-5-carboxy-6-methyl-, | 0.30 1.20 | 0.30 1.20 | 0.30 0.75-0.90 | 0.05-0.10 0.90-1.20 | 0.10-0.20 1.20-1.50 | — 0.90-1.20 | NaOH NaOH |

^a First member in each pair of data represents maximum % concentration tested that permitted growth while the second member represents minimum % concentration that inhibited or killed.

cate that the toxicity of this compound is such that its use may be feasible in certain food industries (8,9).

More detailed studies were made on the bactericidal activity of dehydroacetic acid because of its potential usefulness. Its germicidal activity was determined at different pH ranges and in the presence of organic matter. This compound was also tested in the presence of added thioglycolate to determine if the thiol group had any effect on its bactericidal properties. A summary of these results is included in Table III.

TABLE III
Bactericidal Activity of Dehydroacetic Acid Under Varying Conditions

| Test conditions | Maximum concentration (%) tested that permits growth* | | Minimum concentration (%) tested that is bactericidal | |
|---------------------------------------|--|-------------------|--|-------------------|
| | <i>M. pyogenes</i> var. <i>aureus</i> | <i>S. typhosa</i> | <i>M. pyogenes</i> var. <i>aureus</i> | <i>S. typhosa</i> |
| pH 5.0 | 0.2 | 0.2 | 0.3 | 0.3 |
| pH 7.0 | 0.2 | 0.2 | 0.3 | 0.3 |
| pH 9.0 | 0.2 | 0.2 | 0.3 | 0.3 |
| Presence 10% horse serum | 0.4 | 0.3 | 0.5 | 0.4 |
| Presence 0.1% sodium thioglycolate | 0.2 | 0.3 | 0.2 | 0.3 |

* Test period—7 days.

Dehydroacetic acid enolizes to give a weak acid, hence at pH 9 the compound exists primarily as the sodium salt. The anion must therefore be about as active germicidally as the undissociated acid. This action is in contrast with that of many true organic acids, which are usually much more active germicidally in an acid pH range.

The presence of organic matter decreases the germicidal effectiveness of dehydroacetic acid in line with the results one obtains with most classes of germicides.

Sodium thioglycolate did not have any effect on the antibacterial properties of dehydroacetic acid under the conditions of these tests. Evidently the activity of the compound is not solely dependent upon its reaction with essential bacterial enzymes containing the thiol group. This observation is in agreement with the results reported by Cavallito and Haskell (10) who found that dehydroacetic acid did not react with the thiol group of cysteine.

Further indirect evidence indicates that the reaction of the *alpha*,

beta-unsaturated ketone structure with an essential enzyme containing a thiol group is probably not involved in this series of pyrones. Hydrogenated maltol in which the unsaturation has been lost is as active as the parent compound, maltol.

SUMMARY

1. A group of sixteen substituted pyrones has been studied for antimicrobial activity. 5-Hydroxy-2-(chloromethyl)-1,4-pyrone inhibited the test organisms: *Aerobacter aerogenes*, *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa*, *Penicillium digitatum*, *Rhizopus nigricans*, and *Saccharomyces cerevisiae* in the range of 0.02–0.07%. Dehydroacetic acid (3-acetyl-6-methyl-1,2-pyran-2,4(3*H*)dione) inhibited these organisms in the range of 0.02–0.2%. The remaining pyrones tested have less antimicrobial activity or are so insoluble that tests cannot be made.

2. Evidence has been obtained to indicate that the bacteriostatic properties of hydrogenated maltol and dehydroacetic acid are not dependent primarily upon the reaction of these substituted pyrones with essential bacterial enzymes containing the thiol group.

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Properties of Water-Extractable Apyrases from Different Tissue Sources¹

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INTRODUCTION

Adult frog tissues contain at least two different apyrase-protein fractions, one water-soluble, one soluble in 0.6 *M* KCl, both of which split phosphate from adenosine triphosphate (ATP). Depending on the tissue source, these two fractions are present in different relative proportions (1). The present investigation is confined to a study of the water-extractable apyrases (adenosinetriphosphatases) from different tissues of the frog, and of chicken blood, in an effort to determine whether this enzyme system exhibits different properties from tissue to tissue. The study was prompted by a primary interest in cell differentiation, the process whereby cells become different as the embryo develops into the adult. Apyrase activity is associated with developing frog embryos (2) and apparently, in ascidians, changes occur in this enzyme system which can be correlated with cell differentiation (3). It is of interest to determine whether adult differentiated tissues differ in the properties of the water-soluble enzyme.

METHODS

Apyrase preparations were prepared as previously described (1), 1 g. of tissue homogenized for every 10 ml. extractant (distilled water). One ml. Rohm and Haas ATP (0.0046 *M*) for 0.25 ml. enzyme was added to all experimental tubes, and 0.25 ml. of the desired buffer, the controls receiving ATP after incubation and precipitation with

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0.5 ml. 75% trichloroacetic acid. The tubes were incubated for 5 minutes at the enzyme's temperature optimum of activity, *i.e.*, 45°C., action was stopped with trichloroacetic acid, the solutions were centrifuged, and the supernatants were neutralized and analyzed for inorganic phosphorus by the Fiske-SubbaRow method (4).

The Rohm and Haas ATP was used without further purification, and a new commercial sample was utilized every 4 weeks over the 8-month duration of the experiments. Each curve is an average of at least five separate experiments carried out at different times during this total period. These commercial samples are known to be unstable with time and are undoubtedly contaminated with adenosine diphosphate (ADP). For this reason we use the general term "apyrase" rather than "adenosinetriphosphatase," because we are probably measuring the splitting of both phosphates.

All experiments were conducted without the addition of inorganic ions, except in Sec. III of *Results* where the effect of magnesium and calcium are specifically studied.

The following buffers were used: for pH 4-5.8, acetate; for pH 6.3-6.8, succinate; from pH 7-8.2, veronal; and from pH 9.2-10.2, glycine.

Nuclei were separated from cytoplasm in washed chicken red blood cells by the method of Dounce and Lan (5), and from liver cells by the method of Dounce (6). Laking occurred in the red blood cells after treatment for 15 min. with saponin at pH 6.8, and centrifuging for 1 hr. at 3500 r.p.m. brought down the intact nuclei leaving the supernatant without visible particles. Additional centrifugation of the supernatant for 3 hr. at the same speed brought no further material down and the solution looked clear when examined microscopically. To separate nuclei from cytoplasm in liver cells, frozen liver was homogenized in the presence of citric acid, and after straining the nuclei were separated by differential centrifugation, washed, and resuspended in distilled water.

In the dialysis experiments, the enzyme preparations were dialyzed against distilled water in a 3° cold room for 18-24 hr. on a rocking dialyzer (7). The dialysate was vacuum distilled at 50°C. until 50 ml. volume remained.

The boiled muscle extract was prepared by heating a portion of the muscle extract for 10 min. in a boiling water bath, centrifuging, and discarding the precipitate.

Two preparations of coenzyme A were tested as activators of apyrase activity: one (referred to as crude co A) a crude liver extract obtained from Dr. David Nachmansohn of Columbia University; the other, a small sample of purified coenzyme A (2.7% pantothenic acid) from Dr. Fritz Lipmann of Harvard University. Both are gratefully acknowledged.

EXPERIMENTAL RESULTS

I. pH-Activity Curves

Figure 1 shows the pH-activity curves of the apyrase obtained from different tissues. Micrograms phosphate split from ATP/100 mg. dry weight of tissue/5 min. incubation at 45°C. are plotted against pH. Depending on the tissue source, the curves differ not only in the relative amount of activity, but in the shape of the curve and in the position of the peaks. For frog liver, peaks are found at pH 4.5 and 9.0; for frog muscle, at 7.4; for frog's eggs, at pH 4.0 and 9.0. We elected to study

chicken red blood cells since we were looking for a tissue of relatively pure cell type, liver and muscle being histological mixtures. Even here, the pH-activity curve was no simpler than the more complex tissues, pH optima of red blood cells appearing at pH 5.5, 7, and 8. Plasma appears without activity on this plot, but actually, when incubated for 2 hr., possesses optimal activity at pH 4.5 and 9 as seen in Fig. 2.

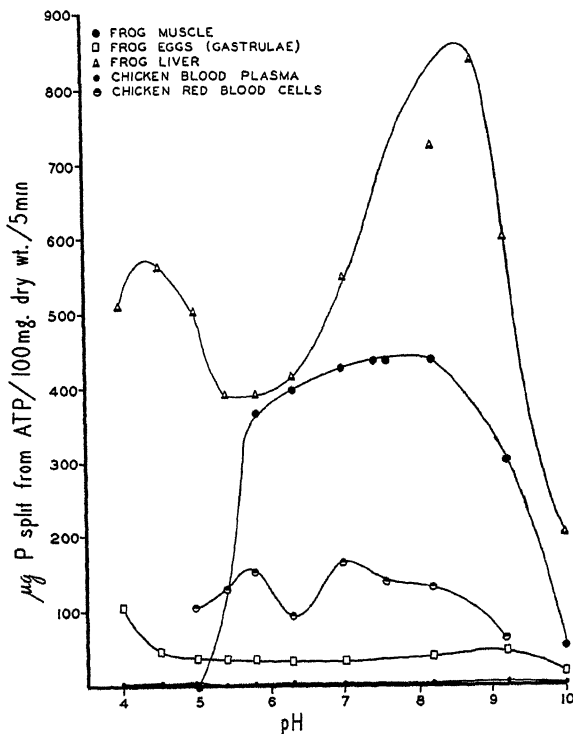


FIG. 1. pH-Activity curves of water-extractable apyrase enzyme from various tissues.

Since some of these peaks are reminiscent of phosphatase peaks, we were concerned with the problem of whether apyrase activity could be distinguished from general phosphatase activity. Consequently, other substrates were substituted for ATP (fructose-6-phosphate, sodium glycerophosphate (20% *alpha*), glyceric acid, phosphate, glucose 1-phosphate, and fructose 1,6-diphosphate). With chicken blood plasma, the

pH-activity curves, using phosphate substrates where the phosphate is attached by an ester linkage, are similar to one another, but somewhat different from the ATP curve, notably in the absence of an acid peak in the former curves. However, the splitting with fructose 1,6-diphosphate exceeds all the others in the alkaline region, including ATP (Fig. 2). These substrates were equimolar with respect to the available phosphate, and present in excess.

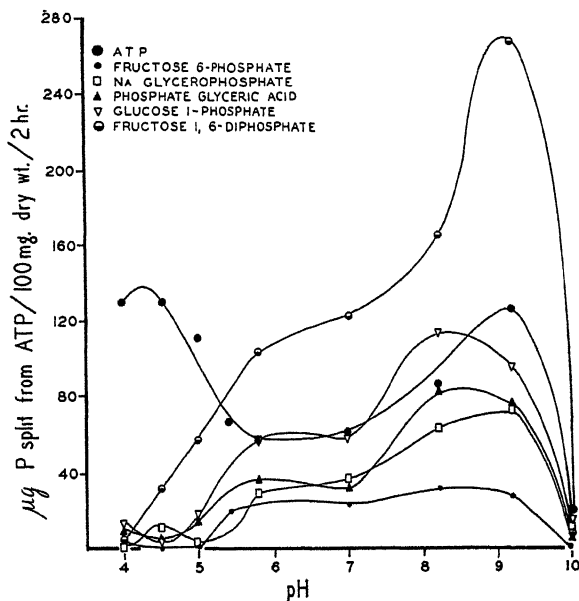


Fig. 2. pH-Activity curve of apyrase activity of chicken blood plasma compared with activity when other phosphate substrates are substituted.

The same substrates gave small activity with frog liver homogenate as compared with ATP as substrate and differed from the ATP curve in lacking an acid peak, with the exception of fructose 1,6-diphosphate (Fig. 3). We interpret these results on pH-activity curves with different substrates to mean that, while phosphatase activity is present in these tissues, on the addition of ATP the liberated phosphate is due to apyrase activity.

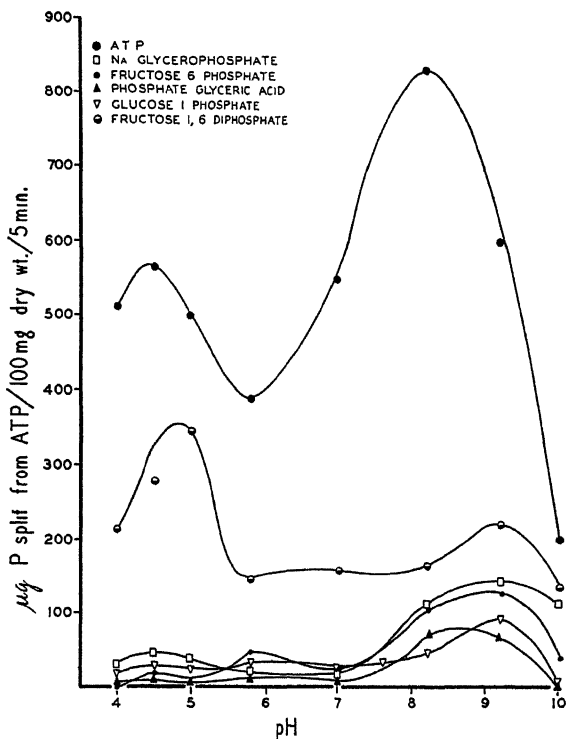


FIG. 3. pH-Activity curve of apyrase activity of frog liver compared with activity when other phosphate substrates are substituted.

II. Localization of Enzyme Activity Within the Cell

In studying the red blood cells of different animals, we noted a correlation between the amount of apyrase activity and the presence or absence of a cell nucleus, as seen in Table I.

TABLE I

The Apyrase Activity of Nucleated and Nonnucleated Blood Cells

| Type of blood | Nucleated | P/100 mg. dry wt./5 min. μg. P |
|---------------|-----------|--------------------------------------|
| Chicken | + | 163.2 |
| Frog | + | 43.9 |
| Mouse | — | 17.6 |
| Human | — | 5.9 |

Consequently, we separated nuclei from cytoplasm in chicken red blood cells, and found most of the activity to be associated with the nuclei (Fig. 4). On an area basis, 90% of the activity was associated with the nucleus, 10% with the cytoplasm. The pII curve for nuclei shows a slight shift and the relative heights of the peaks do not correspond exactly with those of the whole red blood cells, but this is probably due to the drastic treatment of cell breakage.

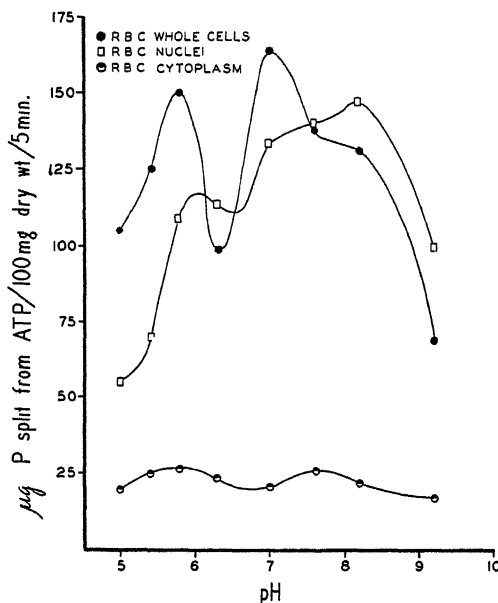


Fig. 4. Apyrase activity of nuclei and cytoplasm of chicken red blood cells as compared with whole red blood cells.

When nuclei were separated from frog liver cells, the apyrase activity was found to be associated with the cytoplasmic region, 10% or less with the nuclei, the exact reverse of the situation with chicken red blood cells. No pH curve of this activity was studied since it was not feasible to obtain sufficient quantities of nuclei. Schneider (8) and Novikoff (9) also found the major portions of the apyrase activity in the cytoplasmic fraction of rat liver cells.

III. The Effect of Calcium and Magnesium on Apyrase Activity

Magnesium stimulates the apyrase activity of chicken blood plasma but does not change the position of the peaks as shown in Fig. 5. With frog liver apyrase, however, magnesium stimulates but to a greater extent at neutral pH's than at acid or alkaline pH's (Fig. 6). Thus the shape of the pH-activity curve is completely distorted in the presence of magnesium. Calcium behaves essentially as magnesium does. With muscle apyrase, calcium and magnesium behave antagonistically, calcium inhibiting and magnesium stimulating the water-extractable apyrase (10). We did not study the effect of these ions on the muscle extract

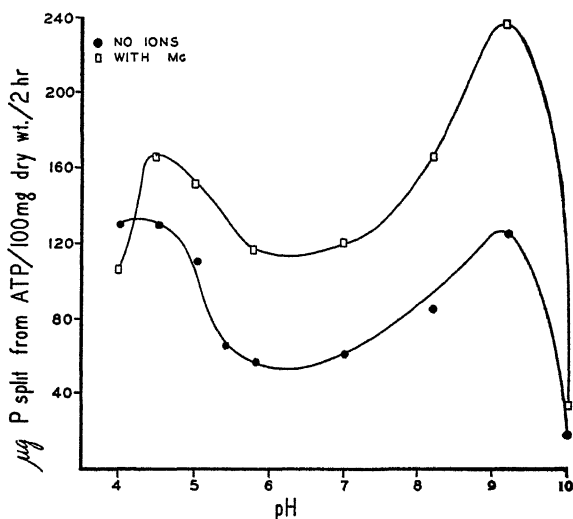


FIG. 5. The effect of magnesium (0.0005 *M* final concentration) on the apyrase activity of chicken blood plasma.

over a pH range. When calcium is added to the apyrase enzyme from frog's eggs, the calcium stimulates, but accentuates the alkaline peak and obscures the acid portion of the curve (Fig. 7).

IV. Dialysis of Water-Extractable Apyrase from Muscle

The water-soluble apyrase from muscle partially loses its activity on dialysis in the cold against distilled water. After 24 hr. dialysis, a loss

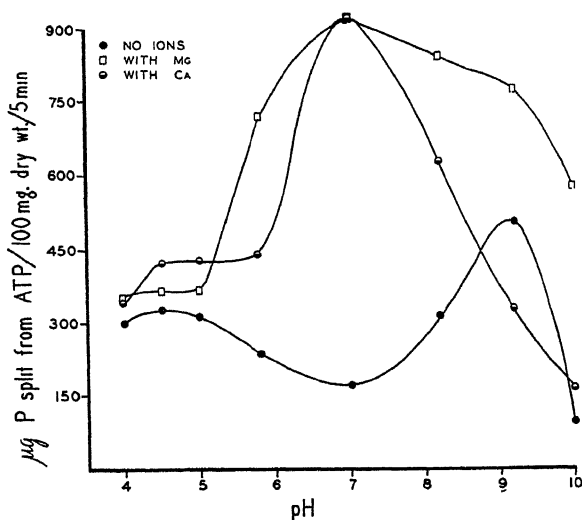


FIG. 6. The effect of magnesium (0.0005 *M* final concentration) and calcium (0.0005 *M* final concentration) on the apyrase activity of frog liver.

of 48% results, an average of 20 determinations. On prolonged dialysis, the loss becomes greater; for example, one enzyme preparation, dialyzed for 60 hr., lost 86% of its activity. Restoration of the loss can be accomplished by reintroduction of the dialysate, concentrated by evapora-

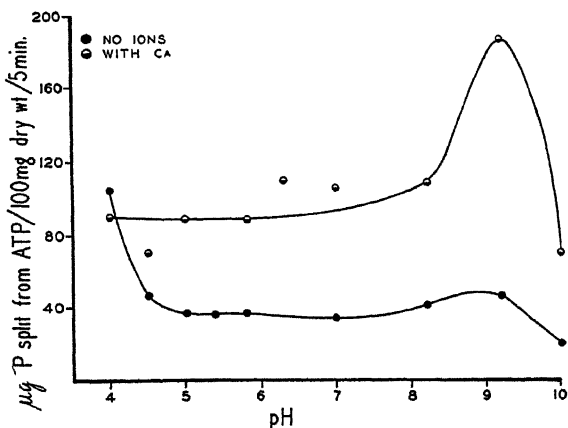


FIG. 7. The effect of calcium (0.0005 *M* final concentration) on the apyrase activity of frog eggs.

TABLE II

The Effect of Dialysis, Dialysate and Boiled Muscle Extract on Water-Soluble Apyrase from Frog Muscle

| Treatment | P split from ATP/100 mg dry wt./5 min. | Increase | Decrease |
|--|--|----------|----------|
| | μg P | per cent | per cent |
| Undialyzed muscle apyrase | 857 | | |
| Dialyzed muscle apyrase | 457 | | 46 |
| Dialyzed apyrase $\frac{3}{4}$ evap. dialysate | 1085 | 137 | |
| Dialyzed apyrase $\frac{3}{4}$ ashed dialysate | 422 | | 8 |
| Dialyzed apyrase $\frac{3}{4}$ boiled extract | 1052 | 130 | |
| Dialyzed apyrase $\frac{3}{4}$ ashed boiled ext. | 171 | | 65 |

TABLE III

The Effects of Various Activators Alone and in Combination on Apyrase Activity of Frog Muscle

Contents of each tube: 0.25 ml. enzyme, 0.25 ml. buffer (veronal at pH 7.4), glutathione (initial concentration 0.04 *M*), or $MgCl_2$ (initial concentration 0.01 *M*), or crude coenzyme A (100 mg. capsule dissolved in 100 ml. distilled water) brought up to total volume of 4.0 ml.

| Activator | Activation | Activation expected if effect is additive |
|--|------------|---|
| | per cent | per cent |
| Enzyme + 0.4 ml. glutathione | 16 | |
| Enzyme + 0.8 ml. glutathione | 66 | |
| Enzyme + 1.2 ml. glutathione | 75 | |
| Enzyme + 1 ml. coenzyme A | 125 | |
| Enzyme + 0.4 ml., glutathione + 1 ml. co A | 91 | 141 |
| Enzyme + 0.8 ml. glutathione + 1 ml. co A | 183 | 191 |
| Enzyme + 1.2 ml. glutathione + 1 ml. co A | 333 | 200 |
| Enzyme + 0.1 ml. $MgCl_2$ | 660 | |
| Enzyme + 0.5 ml. $MgCl_2$ | 380 | |
| Enzyme + 0.5 ml. boiled extract | 260 | |
| Enzyme + 1 ml. boiled extract | 350 | |
| Enzyme + 0.1 ml. $MgCl_2$ + 0.5 ml. boiled extract | 830 | 940 |
| Enzyme + 0.5 ml. $MgCl_2$ + 0.5 ml. boiled extract | 550 | 640 |
| Enzyme + 0.1 ml. $MgCl_2$ + 1 ml. boiled extract | 680 | 1010 |
| Enzyme + 0.5 ml. $MgCl_2$ + 1 ml. boiled extract | 530 | 730 |

tion. However, the ashed dialysate fails to reactivate. Boiled muscle extracts also activate the enzyme, and ashed boiled muscle extracts inhibit the activity. The results of a typical experiment are shown in Table II.

A crude preparation of coenzyme A strongly activates the water apyrase, but a purer sample of co A (higher pantothenic acid content) activates to a lesser extent than the crude. This indicates that some impurity in the crude coenzyme A is the active agent, and not the pantothenic acid derivative present in the preparation.

Magnesium ions, glutathione, cysteine, and histidine also activate the enzyme. But if the enzyme preparation is first saturated with one of these activators and then the boiled extract or co A introduced, the latter preparations continue to activate. Table III shows the result of such an experiment. When two activators are introduced simultaneously, both continue to activate, not always in an additive way, but independently enough to suggest that an unidentified cofactor is present in the boiled extract and co A preparations.

DISCUSSION

Water-soluble extracts from various tissue sources all possess the ability to split phosphate from ATP, but differ markedly in such properties as pH-activity curves, localization of enzyme activity within the cell, and effect of ions such as calcium and magnesium at different pH's. These results point up an important methodological lesson in that they serve to emphasize the fallacy of comparing on a quantitative basis the apyrase activity of different tissues. Unless the complete pH-activity curve for each tissue is known, as well as the effect of activating ions throughout the pH range, one cannot make valid quantitative judgments about relative activities.

A number of workers have studied the water-soluble apyrase activity of different tissues (11-16). Always the apyrase activity was measured at one pH, usually around neutrality, and quantitative comparisons were made of the relative activity found from tissue to tissue. These studies have interest in that they show the relative apyrase activity in tissues at physiological pH's, but they do not mean that the tissue having the highest activity at neutrality is necessarily the most active tissue. For example, DuBois and Potter (11) found in the rat, and Moog (14), in the chick, that the apyrase activity of liver was lower than muscle at neutral pH. In the frog we have shown that neutrality is a

point of low activity for liver and of optimum activity for muscle, the optimum pH of activity for liver being at 9. In reality, frog liver is a more active tissue than muscle with respect to water-soluble apyrase activity, as an examination of the complete curves reveals, a fact which would have been obscured at neutrality. Perhaps the same would be true in the rat and chick, if the pH-activity curves of muscle and liver were studied.

Similarly, if one wishes to compare the relative activity of one apyrase fraction with another within the same tissue, one must know the pH-activity curves of both for a proper comparison. In our earlier work (1) we compared the relative apyrase activity of the water extract and the 0.6 M KCl extract in a number of tissues by assuming the pH characteristics found for muscle to hold in all tissues (optimum of pH 7.4 for the water-soluble fraction and 9.2 for the KCl fraction). The present results show that such an assumption was unwarranted. Indeed, pH curves of the KCl fraction in tissues other than muscle have not been studied, but it is possible that even this extract might exhibit differences from tissue to tissue.

What has been said for pH curves holds for the effect of activating ions such as calcium and magnesium. The effect of these ions in some tissues has been shown here to be discontinuous with pH, and may explain some of the discordant results reported in the literature with calcium activation of water-soluble apyrases, particularly in embryonic tissues (12,14). Perhaps, in these tissues calcium does not activate at the pH chosen for the particular study.

Although each tissue studied here exhibits different properties of its water-soluble apyrase, one cannot assume the presence of a distinct enzyme in each case, since no attempts at purification were made. There is no doubt, however, that the water-soluble apyrase reflects biochemical differences which exist from tissue to tissue. This type of study cannot in itself answer the question of whether these differences are causally related to cell differentiation, but it is hoped that this information, together with that from other approaches, will ultimately add up to an elucidation of the fundamental problem of cell differentiation.

SUMMARY

1. Water extracts of frog liver, muscle, eggs, and chicken red blood cells and blood plasma possess adenosine triphosphate (ATP)-splitting ability but differ markedly in their pH-activity curves.

2. Fructose 6-phosphate, sodium glycerophosphate, glyceric acid phosphate, glucose-1-phosphate, and fructose 1,6-diphosphate are split to a lesser extent than ATP under identical experimental conditions and the pH-activity curves differ from that with ATP, using extracts from frog liver. With chicken blood plasma, the same results are found, with the exception of fructose 1,6-diphosphate.

3. Nucleated red blood cells of the chicken and frog possess much higher apyrase activity than nonnucleated red cells of the mouse and human.

4. Separating the nucleus from the cytoplasm in chicken red blood cells reveals that 90% of the apyrase activity is associated with the nucleus. The reverse was found to be true on separation of nuclei from cytoplasm in liver cells. Here, 90% of the activity is found in the cytoplasmic fraction, 10% or less in the nuclei.

5. Magnesium and calcium behave differently with extracts from different tissue sources. Magnesium stimulates the activity of the chicken blood plasma uniformly throughout the pH curve; it stimulates the frog liver apyrase discontinuously with pH, activating more at neutral pH's than at the acid and alkaline regions. Calcium behaves essentially like magnesium on the frog liver apyrase. The apyrase from frog's eggs is stimulated by calcium, but primarily in the alkaline region, thus obscuring the acid peak.

6. Dialysis of the water-soluble apyrase from muscle results in partial loss of activity, which can be restored on reintroduction of the dialysate concentrated by evaporation. The ashed dialysate fails to reactivate the apyrase.

7. Boiled muscle extract also activates the water apyrase from muscle, and ashing destroys this ability. Crude coenzyme A activates the apyrase enzyme, and a purer sample of coenzyme A (higher pantothenic acid content) to a lesser extent. Apparently, some unknown, organic, dialyzable cofactor stimulates activity; it does not appear to be magnesium, glutathione, cysteine, or histidine, all of which are known to activate the enzyme.

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Preparation of D-Glutamic Acid from DL-Glutamic Acid by a Decarboxylase Method ¹

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INTRODUCTION

The action of specific bacterial decarboxylases on DL-amino acids might be expected to yield products from which the corresponding D-amino acids could easily be isolated, since these enzymes attack only L-amino acids (2). The preparation of D-lysine from DL-lysine by such a decarboxylase method has been described by Neuburger and Sanger (3). A similar method, described in the present paper, has been developed in the authors' laboratory for the preparation of high quality D-glutamic acid from the DL-compound. Although D-glutamic acid has been prepared previously by enzymatic (4,5) and other (6-12) means, the present method appears to have the advantages of directness and simplicity.

EXPERIMENTAL

L-Glutamic Acid Decarboxylase

Dried cells of *Escherichia coli*,² prepared by a modification of the method of Umbreit and Gunsalus (13)³ were found to possess satisfactory activity for the purpose of the present experiments.

¹ Paper No. 70. For Paper No. 69, see Murphy and Dunn (1). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council, Swift and Company, and the University of California.

² Strain 86M, American Type Culture Collection, catalog number 4157.

³ The authors are indebted to Dr. M. J. Blish, International Minerals and Chemical Co., Rossford, Ohio, and Dr. Lucile Hac, International Minerals and Chemical Co., Woodland, California, for supplying a sample of dried cells for use in the present experiments.

Preparation of D-Glutamic Acid

The following procedure was the most satisfactory of those tested in the present experiments. Thirty grams of DL-glutamic acid⁴ was dissolved in 400 ml. of water and the mixture was adjusted to pH 5.0 with the addition of 12.8 ml. of saturated ammonium hydroxide solution. Six grams of decarboxylase powder was added and the mixture was stirred slowly at room temperature with a motor stirrer. A few drops of *n*-octyl alcohol were added to avoid excessive foaming from carbon dioxide evolution. The pH of the mixture rose as the reaction proceeded, and 2 *N* hydrochloric acid was added at intervals in amounts sufficient to bring the pH to 5.0. When the pH became constant (after about 4 hr.), the reaction was assumed to be complete. The reaction mixture was diluted to about 3 l. with water and clarified by passing it through a Sharples supercentrifuge.⁵ The supernatant solution was acidified with 500 ml. of 12*N* hydrochloric acid and evaporated to dryness under reduced pressure, and the resulting solids were dissolved in 150 ml. of water. This solution was filtered and saturated with hydrochloric acid gas while cooling in an ice bath. D-Glutamic acid hydrochloride crystallized spontaneously from the reaction mixture, which was allowed to stand overnight in a refrigerator. The crystals were collected on a sintered-glass filter, washed successively with three 50-ml. portions of ethanol-ether mixture (1:1), and dried at 50° for 6 hr. The yield was 18 g. of D-glutamic acid hydrochloride, approximately 96% of the theoretical amount.

The following procedure was employed to obtain free D-glutamic acid. Fourteen grams of the D-glutamic acid hydrochloride was dissolved in 150 ml. of water, warmed at 50° with 1 g. of Norite-A, and filtered. The filtrate was adjusted to pH 3.2 with ethanolamine and cooled in an ice bath. Crystallization was induced by scratching the walls of the container with a glass rod, and the mixture was allowed to stand overnight in the refrigerator. The crystals were collected on a filter, washed successively with two 25-ml. portions of ethanol (absolute) and two 25-ml. portions of ethanol-ether mixture, and dried at 50° for 6 hr. The yield was 7.9 g. (70.5% of theory) of pure white product. The specific rotation was $[\alpha]_D^{25} = -31.41^\circ$ in 1.7 *N* hydrochloric acid.

⁴ C.P. Product of Amino Acid Manufactures.

⁵ It was found possible to reuse the decarboxylase recovered in the supercentrifuge, although the reaction with the recovered enzyme proceeded at a much lower rate (complete in less than 24 hr.).

Several batches of D-glutamic acid (total of 21.1 g.) were ground to a fine powder and rapidly dissolved in water which had been preheated to 80°. The solution was immediately filtered through hard paper and rapidly cooled in an ice bath. The flask was shaken frequently during the first 4 hr. of crystallization to prevent caking, and the mixture was allowed to stand overnight in the refrigerator. The crystals were collected on a filter and washed successively with two 25-ml. portions of ice water, three 50-ml. portions of absolute ethanol, and three 50-ml. portions of ether, and dried at 50° for 6 hr. The yield of recrystallized D-glutamic acid was 18 g. (85% recovery). The specific rotation was $[\alpha]_D^{25} = -31.71^\circ$ in 1.7 *N* hydrochloric acid.

DISCUSSION

The progress of the decarboxylation reaction was easily followed by measuring the amounts of standard acid added to bring the reaction mixture to pH 5.0. This is illustrated by the data from a typical decarboxylation experiment which are plotted in Fig. 1. It may be seen (Fig. 1) that the rate of the reaction fell off slightly during the course of the decarboxylation, and dropped rapidly to zero at the end of the reaction. The standard acid used was equivalent ($95 \pm 1\%$) to the L-glutamic acid initially present in the reaction mixture as found in a number of quantitative tests with small amounts (10–80 mg.) of analytically pure L- and DL-glutamic acids. This relatively constant relationship between the L-glutamic acid present and the standard acid required suggests the possibility of a titrimetric decarboxylase method (in place of the manometric methods now being used) for the determination of L-glutamic acid. Such titrimetric decarboxylase methods are being investigated in the authors' laboratory.

Microbiological assays of decarboxylated L-glutamic acid samples indicated that less than 0.2% of the L-glutamic acid initially present escaped decarboxylation. That the decarboxylase reaction proceeded nearly to completion was also apparent from the high purity of the D-glutamic acid which was obtained with one recrystallization. The specific rotation ($[\alpha]_D^{25} = -31.71^\circ$) indicated that the recrystallized product contained no significant amount of L-glutamic acid, since this rotation is equal and opposite to that of analytically pure L-glutamic acid (14).

In other laboratories D-glutamic acid has been obtained by resolution of the strychnine salt of benzoyl-DL-glutamic acid (6), by resolution of

the quinine salt of DL-pyroglutamic acid (7,8), by the action of molds (9-11) and yeast (12) on DL-glutamic acid, and by the action of papain-cysteine on carbobenzoxy-DL-glutamic acid anilide (4,5). D-Glutamic acid has also been prepared by hydrolysis of the polyglutamic acid produced by *Bacillus subtilis* [H. S. Olcott, quoted by Dunn *et al.*,

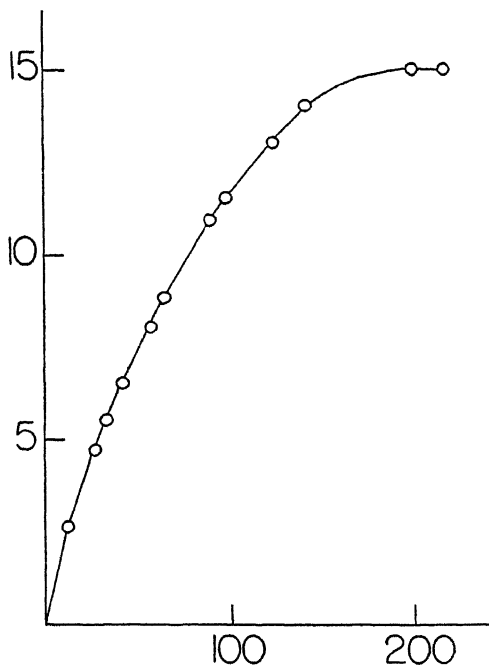


FIG. 1. Reaction of 10 g. of DL-glutamic acid with 2.5 g. of L-glutamic acid decarboxylase. The total volumes (in milliliters) of 2 *N* acid (values on vertical scale) added to bring the pH of the mixture to 5.0 have been plotted against the intervals of time in minutes (values on horizontal scale) at which the additions were made.

(15)], although it appears that details of this preparation have not been published.

SUMMARY

A method has been described for the preparation of D-glutamic acid by isolating it as the hydrochloride from the reaction mixture obtained by the action of a specific L-glutamic acid decarboxylase on DL-glutamic acid.

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The Significance of Dietary Pyridoxine, Niacin and Protein in the Conversion of Tryptophan to *N*¹-Methylnicotinamide

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INTRODUCTION

The transformation of tryptophan to nicotinic acid in rats (6,12), horses (11), man (4,8,9), and other species has been demonstrated. It had been shown that the urinary excretion of *N*¹-methylnicotinamide (MN) by rats on pyridoxine-deficient diets gradually decreased, and that, simultaneously, the excretion of xanthurenic acid (XA) increased, after tryptophan administration (3,5,10). The failure of pyridoxine to restore the ability of these rats to convert tryptophan to MN pointed to some metabolic impairment produced during the period of vitamin deprivation (5). Since previous experiments with unbalanced proteins also indicated some impairment of this conversion (7), it was thought that the amino acid unbalance caused by pyridoxine deficiency, especially on high protein diets, would also lead to the failure of the conversion of tryptophan to MN. It was therefore of interest to determine whether pyridoxine deficiency, *per se*, or the protein level of the diet, was the critical factor in this conversion.

EXPERIMENTAL

Weanling rats (male) of the Vanderbilt strain were housed in individual metabolism cages with wire-mesh bottoms. These animals were divided into 9 groups of 3 rats each. The basal diet consisted of casein 10 g., sucrose 82 g., cottonseed oil 3 g., salts 4 g. (1), choline 0.5 g., and L-cystine 0.4 g./100 g. of diet. Higher levels of casein were added at the expense of sucrose. Each kilogram of basal diet was supplemented with thiamine 10.0 mg., riboflavin 10.0 mg., Ca pantothenate 60 mg., inositol 156 mg., and

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vitamin K 1.0 mg., respectively, plus biotin 200 μ g., and folic acid 200 μ g., respectively. Two drops of cod-liver oil was given twice weekly. L-Cystine was omitted and choline reduced to 0.2 g./100 g. of diet at the 30% and 50% casein levels. Each animal was supplied with 10 g. of food daily, and was weighed twice weekly. Any excess food remaining in the cups was mixed daily with sufficient fresh diet to make a total of 10 g. The experiment lasted 8 weeks, during which time doses of 100 mg. DL-tryptophan were administered orally to each animal, at approximately weekly intervals, by mixing the amino acid with the diet. After 7 weeks, the protein was removed from all diets, and was replaced at each level by an equal weight of sucrose for a period of 7 days. Individual 48-hr. urine samples were collected over 1 ml. toluene and 0.5 ml. of glacial acetic acid, filtered, and adjusted to a final volume of 50.00 ml. from which suitable aliquots were withdrawn for analysis. Control values were obtained by analysis of the urine samples taken immediately preceding the 1st, 2nd, and 5th periods of tryptophan administration.

The urinary excretion of *N* ¹-methylnicotinamide was determined on the individual samples by the fluorometric acetone condensation method of Huff and Perlzweig (2), and xanthurenic acid was measured colorimetrically by a procedure based on the formation of a green ferric salt in an alkaline medium (5). The quinolinic acid fraction was measured in the pooled group urines. One to 3 ml. urine was evaporated to dryness, autoclaved for 15 min. at 15 lb. pressure with 3 ml. of glacial acetic acid, adjusted to pH. 6.8-7.0, and made up to a suitable volume (ca. 200 ml.) with distilled water. Samples were removed for microbiological determination of nicotinic acid employing *Lactobacillus arabinosus* 8014.

RESULTS AND DISCUSSION

The production of MN in response to a dose of tryptophan by pyridoxine-deficient rats on a 10% protein diet gradually decreased during the progress of the experiment (Table I). At this level, the inclusion of pyridoxine in the diet prevented this decrease, and in the absence of pyridoxine, niacin stimulated MN production. Within any given collection period, the excretion of MN was greater in the group receiving 10% casein rather than 30%, and was also higher at the 30% than 50% protein levels, but these differences tended to disappear shortly after the removal of the protein from the diet. Thus, in contrast to previous data obtained in these laboratories (5), and elsewhere (10), under the experimental conditions described in this report, pyridoxine does not appear to be directly involved in the transformation of tryptophan to *N* ¹-methylnicotinamide in the rat.

Dramatic responses to tryptophan feeding were noticed (as increases in MN excretion) after removal of the protein component from the diet. Nicotinic acid added to B₆-deficient diets stimulated MN production to a level comparable to that of the animals receiving pyridoxine.

That the transformation of tryptophan to MN by rats on the 50% protein diet was so poor before all protein had been removed indicates an impairment or blocking of the mechanisms involved in this conversion. The anomalous behavior of the rats on the 50% protein diet plus 4 mg.-% pyridoxine, following the removal of the protein from the diet, might be explained on the basis of a more severe impairment from which these rats recover more slowly than do rats on comparable diets of lower protein content. Support for this is found in the MN excretion

TABLE I

Average of Individual N¹-Methylnicotinamide Excretion in 48-Hr. Urine Samples After Administration of 100 mg. DL-Tryptophan

The values shown are increments over comparable controls in $\mu\text{g.}/48 \text{ hr.}$

| Casein level | Group ^a | Collection periods | | | | | |
|--------------|--------------------|--------------------|------|------|------|--------------------|------|
| | | Proteins present | | | | Proteins withdrawn | |
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| % | | | | | | | |
| 10 | 1 | 2359 | 2042 | 1270 | 450 | 690 | 504 |
| 10 | 2 | 2363 | 1433 | 2511 | 2488 | 1536 | 1866 |
| 10 | 3 | 2068 | 1915 | 1420 | 1090 | 1627 | 1897 |
| 30 | 4 | 753 | 733 | 849 | 641 | 940 | 1140 |
| 30 | 5 | 727 | 460 | 854 | 547 | 1146 | 1826 |
| 30 | 6 | 467 | 476 | 674 | 672 | 1604 | 1684 |
| 50 | 7 | 484 | 203 | 196 | 267 | 688 | 1228 |
| 50 | 8 | 175 | 451 | 232 | 186 | 407 | 807 |
| 50 | 9 | -27 | 229 | 153 | 130 | 822 | 1742 |

^a Groups: 1, 4, 7 received no supplement to diet; 2, 5, 8 received 4 mg.-% pyridoxine-HCl; 3, 6, 9 received 5 mg.-% nicotinic acid.

data for the fifth and sixth collection periods. Although there is little difference between the amounts of MN excreted at the 10% and 30% casein levels, the values double during the same interval for the rats previously receiving a 50% protein diet. This may indicate that a longer period of protein deprivation is necessary to restore the capacity of these rats to effect this conversion.

The excretion of MN, by pyridoxine-deficient rats fed tryptophan,

increased upon protein withdrawal, but the rats were unable to synthesize MN, as indicated by excretion data, in as large quantities as B₆-deficient animals also receiving nicotinic acid. This is in agreement with the previous observation, that if B₆-deficient animals were given pyridoxine, and fed tryptophan, the XA production decreased, whereas the MN excretion could not be increased to its previous "normal" level (5). The ability of relatively small amounts of dietary nicotinic acid to correct this situation in the absence of protein is evident.

TABLE II

Average of Individual Xanthurenic Acid Excretions in 48-Hr. Urine Samples After Administration of 100 mg. DL-Tryptophan

The values shown are increments over comparable controls in $\mu\text{g.}/48 \text{ hr.}$

| Group no. | Collection periods | | | | | |
|-----------|--------------------|--------|-------|-------|-------------------|-------|
| | Protein present | | | | Protein withdrawn | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 3,995 | 1,883 | 2,043 | 2,545 | 4,450 | 6,893 |
| 2 | 840 | 1,306 | 946 | 76 | 542 | 297 |
| 3 | 1,737 | 4,637 | 1,705 | 1,143 | 1,056 | 470 |
| 4 | 2,283 | 7,607 | 3,696 | 4,182 | 6,550 | 6,083 |
| 5 | 255 | 582 | 255 | 172 | 388 | 322 |
| 6 | 1,923 | 7,620 | 2,657 | 2,090 | 1,460 | 892 |
| 7 | 2,236 | 11,123 | 5,373 | 3,746 | 2,993 | 4,160 |
| 8 | 673 | 512 | 344 | 9 | 668 | 307 |
| 9 | 2,489 | 6,560 | 1,547 | 1,412 | 1,490 | 825 |

It was observed that the XA excretion by the rats receiving neither pyridoxine nor niacin after a dose of 100 mg. DL-tryptophan was greater than in those rats receiving niacin only, whereas the excretion of XA by the rats receiving B₆ was as expected, much less than that of the other groups (Table II). This general trend was observed at all levels of casein, and was more evident after the second collection period of the experiment. Although the data is presented as the mean of individual analyses, the niacin effect was consistent for all the animals within any one group. The XA excretion by pyridoxine-deficient rats

was greater at the 30% and 50% casein levels than at 10% which may be a reflection of the amount of extra tryptophan present in the protein. Apparently, administered nicotinic acid "protects" the rat in some unknown way from the excretion of large amounts of XA on a pyridoxine-deficient diet after administration of tryptophan.

The presence of nicotinic acid in the diet caused a decrease in XA production after removal of protein. This may be correlated with a simultaneous increase of MN excretion by these rats. A similar correlation apparently exists for rats receiving pyridoxine. This decrease of XA excretion caused by added nicotinic acid cannot be ascribed solely to the lower tryptophan content of the diet caused by protein withdrawal, since no decrease is observed in the XA excretion of B₆-deficient animals not receiving nicotinic acid. In general, the magnitude of XA excretion is similar in comparable groups after complete withdrawal of diets.

The quinolinic acid fraction (QAF) includes free nicotinic acid present in the urine before treatment with glacial acetic acid. Pure quinolinic acid added to a urine sample has been recovered as nicotinic acid in almost theoretical amounts. The excretion of the QAF was inversely

TABLE III

*Nicotinic Acid Measured in Pooled Group of 48-Hr. Urine
Samples Autoclaved with Glacial Acetic Acid*

The values shown are increments over comparable controls in $\mu\text{g.}/48 \text{ hr.}$

| Group no. | Collection periods | | | | | |
|--------------|--------------------|-------|-------|-------|-------------------|-------|
| | Protein present | | | | Protein withdrawn | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 4,950 | 3,130 | 2,280 | 304 | 415 | 465 |
| 2 | 4,073 | 3,673 | 3,983 | 1,950 | 5,223 | 2,473 |
| 3 | 4,762 | 1,477 | 3,512 | 1,757 | — | 3,442 |
| 4 | 1,249 | 352 | 333 | 166 | 1,939 | 1,339 |
| 5 | 1,251 | 169 | 1,181 | 171 | 3,571 | 3,141 |
| 6 | 1,304 | 133 | 638 | 279 | 3,034 | 969 |
| 7 | 268 | 52 | 152 | 66 | 438 | 1,084 |
| 8 | 223 | 168 | 380 | 832 | 253 | 537 |
| 9 | 105 | 40 | 192 | —8 | 96 | 702 |

proportional to the dietary protein level (Table III). The excretion increased after protein removal, but this, however, may be a nonspecific effect, since the MN formed from tryptophan also increases. No other comparable relationship is apparent in this experiment between the urinary excretion of MN and QAF.

Inspection of the growth data revealed no significant differences between the groups receiving nicotinic acid or pyridoxine in the maximum weights obtained (*ca.* 132 g.), or in the general slope of the growth curves for the animals receiving 10, 30, or 50% casein. Similarly, no significant differences were evident between growth rates of the rats on the unsupplemented diets, whether on 10, 30, or 50% protein; the maximum weight obtained by these animals, however, was significantly less (*ca.* 110 g.) than when nicotinic acid or pyridoxine was given. These growth data seem to infer that only a mild B₆ deficiency was produced. The restriction of the food supply to the rats may have prevented better growth by the rats receiving pyridoxine or niacin.

The data obtained under the conditions of this experiment indicate that the level at which casein is present in the diet is of primary importance in the conversion of tryptophan to MN in the rat.

SUMMARY

1. Under the conditions of this experiment, the urinary excretion of *N*¹-methylnicotinamide and quinolinic acid by rats, after feeding 100 mg. DL-tryptophan, is inversely proportional to the level of casein in the diet, independent of supplementation by either nicotinic acid or pyridoxine.
2. The increased urinary excretions of xanthurenic acid by pyridoxine-deficient rats after tryptophan feeding is diminished by the addition of nicotinic acid to the diet.
3. Pyridoxine does not appear to be directly involved in the conversion of tryptophan to *N*¹-methylnicotinamide by the rat.

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The Decomposition of Chloromycetin¹ (Chloramphenicol) by Microorganisms²

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INTRODUCTION

Previously it has been observed that certain bacteria or bacterial enzymes are able to decrease the antibiotic potency of chloromycetin solutions by hydrolyzing the amide linkage of the drug (9,10), or by reducing the nitro group to a primary amine (11). These observations suggested the possibility that bacteria sensitive to the antibiotic might be employing a series of reactions either individually or in combination to degrade the drug. Preliminary experiments proved this to be the case and it seemed desirable, therefore, to attempt to isolate and identify the various products formed by bacterial decomposition of chloromycetin.

The present study reveals the nature of the various decomposition products formed. In addition, several possible pathways have been postulated by means of which the drug can be degraded, and the individual differences exhibited by the various bacteria tested in using these pathways have been indicated.

METHODS

The bacterial decomposition of chloromycetin was studied by incubating suspensions of the test organisms with the antibiotic for various periods of time at 37°C. The time periods used were 0, 4, 8, 12, 16, 20, and 48 hr. After incubation, the suspensions were filtered through a Seitz-filter and the filtrate obtained subjected to the liquid-liquid extraction procedure outlined in Fig. 1. Approximately 100 ml. of filtrate was used in each experiment and was extracted with an equal volume of each organic solvent. The various fractions obtained from the extraction procedure were concentrated to approximately 2-3 ml. by lyophilizing and chromatographed according to the procedure previously described (14) on paper sheets 45 × 55 cm. The sheets were

¹ Parke, Davis & Company's trade name for chloramphenicol.

² Reported in part at the thirty-fourth annual meeting of the Federation of American Societies of Experimental Biology, Atlantic City, April, 1950.

then sectioned into 5-cm. strips, the compounds eluted from each section and rechromatographed on paper strips 2×55 cm. These strips were then developed by the various chemical methods (14). The chromatograms were compared with standard chromatograms containing known quantities of the various compounds related to chloromycetin.

The organisms used in these experiments were *Escherichia coli* No. 04839, *Bacillus mycoides* No. 04573, *Proteus vulgaris* No. 0924, and *Bacillus subtilis* No. 0255 obtained from the Parke, Davis & Company Culture Bureau.

The bacteria were grown in an aerated nutrient broth medium and suspensions of the organisms prepared according to the directions given by Smith *et al.*, (13). In these experiments 65 ml. of the bacteria suspension, containing approximately 15 ml. of packed cells in 0.9% neutral saline, was mixed with 35 ml. of chloromycetin solution which contained 3 mg. of the antibiotic/ml. dissolved in the neutral saline solution. This mixture was then incubated as described above and the various decomposition products of chloromycetin isolated from the filtrate obtained from this suspension.

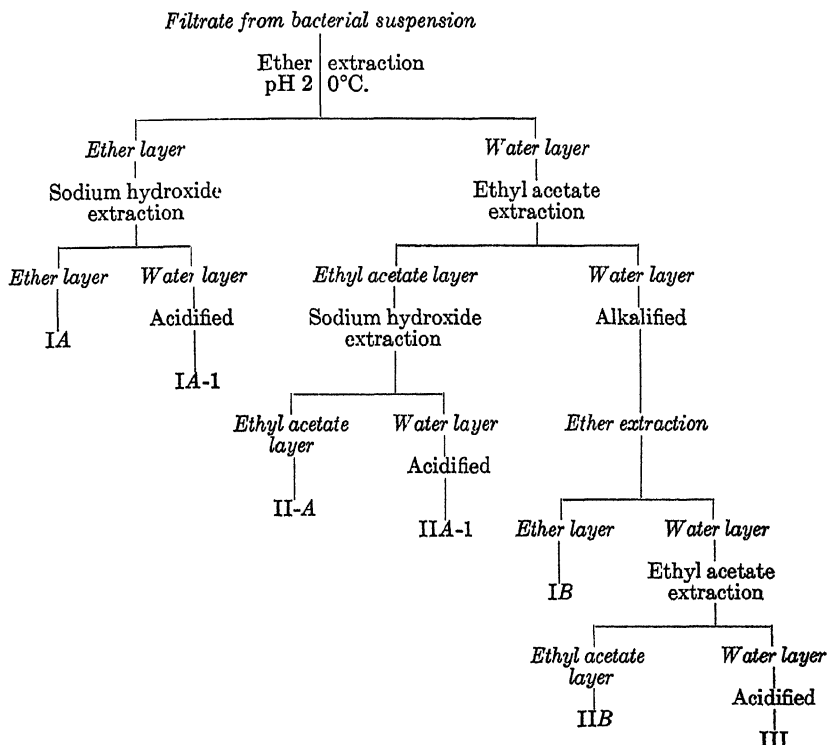


FIG. 1. The separation of the bacterial decomposition products of chloromycetin by liquid extraction.

RESULTS

By using the procedures described above, it has been possible to isolate and identify the various decomposition products of chloromycetin shown in Table I. The quantity of each compound found in the suspension incubated for 48 hr. was roughly estimated by comparison with standard chromatograms and is indicated in the table by asterisks. The term polymers used in this discussion refers to all the high-molecular-weight compounds which are formed by condensation of one or more of the known decomposition products and which precipitate out

TABLE I
*The Various Decomposition Products of Chloromycetin Formed
by the Action of Bacteria on the Antibiotic*

| Compound | Bacteria acting on drug | | | |
|---|-------------------------|--------------------|----------------|--------------------|
| | <i>B. subtilis</i> | <i>B. mycoides</i> | <i>E. coli</i> | <i>P. vulgaris</i> |
| 1- <i>p</i> -Nitrophenyl-2-amino-1, 3-propanediol (4,7) | **** | **** | ** | **** |
| α -Amino- β -hydroxy- <i>p</i> -nitropropionophenone (5,6) | *** | *** | * | ** |
| α -Dichloroacetamido- β -hydroxy- <i>p</i> -nitropropionophenone (5,6) | ** | **** | * | *** |
| <i>p</i> -Nitrobenzaldehyde | **** | **** | | **** |
| <i>p</i> -Nitrophenylserine | ** | ** | * | * |
| <i>p</i> -Nitrobenzoic acid | * | ** | | * |
| 1- <i>p</i> -Aminophenyl-2-dichloroacetamido-1, 3-propanediol (7) | ** | *** | **** | * |
| 1- <i>p</i> -Aminophenyl-2-amino-1,3-propanediol | **** | ** | **** | *** |
| α -Amino- β -hydroxy- <i>p</i> -aminopropionophenone | | | * | ? |
| α -Dichloroacetamido- β -hydroxy- <i>p</i> -aminopropionophenone | | | ? | ? |
| <i>p</i> -Aminobenzaldehyde | | * | ** | * |
| <i>p</i> -Aminophenylserine | * | * | ** | * |
| <i>p</i> -Aminobenzoic acid | | * | ** | ? |
| Ethanolamine | *** | ** | * | * |
| Formaldehyde | ? | ? | ? | ? |
| Ammonia | ? | ? | ? | ? |
| Carbon dioxide | ? | ? | ? | ? |
| Polymers | **** | **** | **** | **** |

of the filtrate on cooling to 0°C. It has not been possible to determine the number of compounds present in this mixture or assign any definite structure to them. In the final stages of the decomposition of chloromycetin solutions, a large percentage of the original quantity of the antibiotic can be accounted for in terms of these polymers. The occurrence of α -dichloroacetamido- β -hydroxy-*p*-aminopropiophenone is still questionable since it has not been possible to obtain sufficient quantities of this compound to permit a chemical elucidation of its structure.

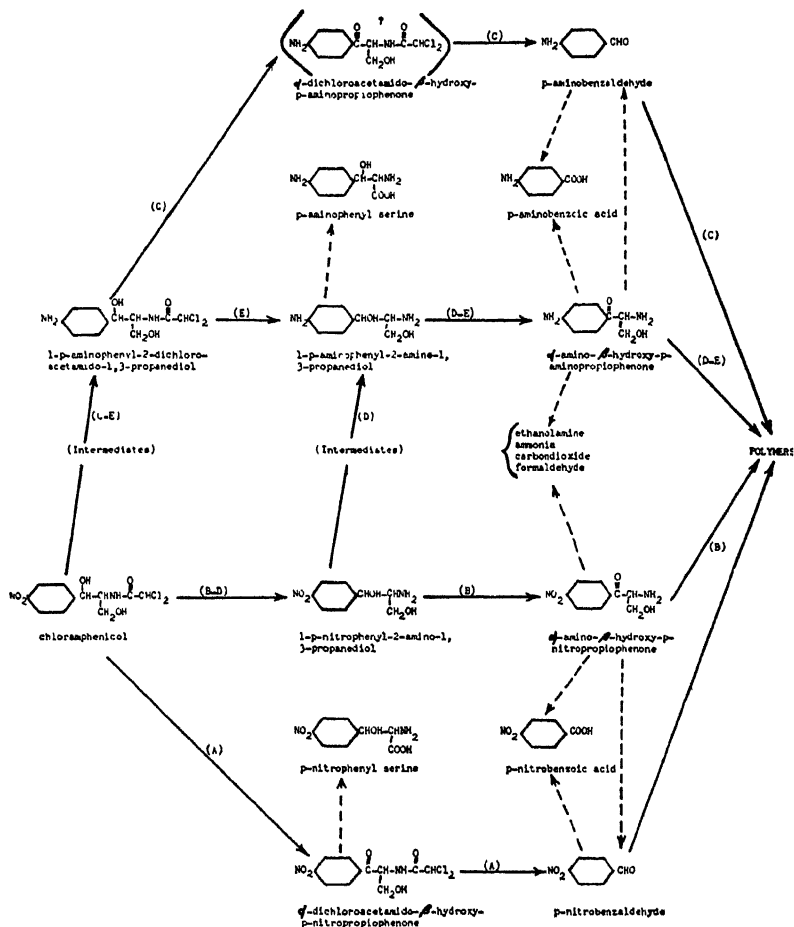
Indications have been obtained to show that formaldehyde, ammonia, and carbon dioxide are formed during the bacterial decomposition of chloromycetin. It has not, however, been possible to establish definitely that these compounds have originated through the breakdown of the propanediol portion of the molecule (8).

DISCUSSION

From a study of the chemical relationships between the various decomposition products formed by the action of bacteria on chloromycetin and how soon each product appeared in the bacterial suspension, it is possible to postulate several routes by means of which the drug might be degraded. These various routes have been diagrammed in Fig. 2. It appears that there are at least 5 pathways possible by means of which chloromycetin can be decomposed. By route *A*, the drug is first oxidized at the secondary hydroxyl group with the formation of a nitropropiophenone derivative. The molecule can then split at this position to give *p*-nitrobenzaldehyde which generally accumulates in the filtrate as either the free compound or in combination.

In pathway *B*, the molecule is first hydrolyzed at the amide linkage presumably by chloramphenicolase (15) to give 1-*p*-nitrophenyl-2-amino-1,3-propanediol which can then be oxidized to form a nitropropiophenone derivative. This latter compound can either be split at the point of oxidation to give nitrobenzaldehyde or nitrobenzoic acid, or can condense to form a cyclic dimer. In the later case, the aliphatic amine group of one molecule combines with the carboxyl group of the second molecule to form a six-member ring. Of the two reactions, the condensation reaction is the predominant one.

If chloromycetin is degraded according to route *C*, the compound is first reduced to an arylamine derivative then oxidized to a *p*-aminopropiophenone compound which splits to form *p*-aminobenzaldehyde which rapidly undergoes polymerization.



The steps involved in pathway *D* consist of hydrolysis followed in turn by a reduction of the nitro group and oxidation of the secondary hydroxyl group. The aminopropiophenone compounds formed by these reactions can either be cleaved to form *p*-aminobenzaldehyde or *p*-aminobenzoic acid, or undergo polymerization which is the more likely reaction.

According to pathway *E*, chloromycetin is first reduced, then hydrolyzed, and finally oxidized to α-amino-β-hydroxy-*p*-aminopropiophenone which can then undergo the reactions described above.

Although the results obtained suggest that each of the four organisms tested is able to use each pathway to some extent in degrading the drug, it is apparent that each organism has a preference as to which pathway or pathways it will employ.

In the case of *Bacillus mycoides*, the large accumulation of 1-*p*-nitrophenyl-2-amino-1,3-propanediol, α -amino- β -hydroxy-*p*-nitropropionophenone, α -dichloroacetamido- β -hydroxy-*p*-nitropropionophenone, 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol, and *p*-nitrobenzaldehyde indicates that decomposition of chloromycetin must be proceeding through pathways *A*, *B*, *D* or *E*. Quantitative studies of the production of arylamine derivatives from chloromycetin by *B. mycoides* reveal that the organism can convert only about 2% of the drug into arylamine compounds. This would eliminate pathways *D* and *E* as being important in the major breakdown of the drug by this organism. The early appearance of 1-*p*-nitrophenyl-2-amino-1,3-propanediol and α -dichloroacetamido- β -hydroxy-*p*-nitropropionophenone, indicates that decomposition must proceed mainly through pathways *A* and *B*. The large accumulation of *p*-nitrobenzaldehyde would designate *A* as the main pathway since there is a much greater possibility of the nitrobenzaldehyde coming from α -dichloroacetamido- β -hydroxy-*p*-nitropropionophenone than from α -amino- β -hydroxy-*p*-nitropropionophenone as has been discussed previously. Apparently a small amount of the α -amino- β -hydroxy-*p*-nitropropionophenone is split, as ethanalamine has been found in the filtrate. Ammonia, carbon dioxide, and indications of formaldehyde have also been detected, but it has not been possible to establish the fact that these compounds have been derived from the breakdown of chloromycetin.

The large accumulation of *p*-nitrobenzaldehyde in suspensions of *Bacillus mycoides* indicates that this compound is the major final product in the breakdown of the drug by this organism. The *p*-nitrobenzaldehyde can be converted to *p*-nitrobenzoic acid, *p*-aminobenzaldehyde, or *p*-aminobenzoic acid which can be detected in small quantities in the filtrate. It appears, however, that *p*-nitrobenzaldehyde largely accumulates in the free state and may even precipitate in the filtrate, especially when the filtrate is cooled to 0°C.

The major pathways apparently employed by *Escherichia coli* for the decomposition of chloromycetin are quite different from those observed in the case of *B. mycoides*. In the initial stages of the breakdown, the loss in antibiotic activity of the suspensions can be accounted for in

terms of the appearance of arylamine compounds. A suspension of *E. coli* can convert approximately 25–30% of the antibiotic into arylamine derivatives in about 24 hr. The first arylamine compound that appears, as revealed by the time interval studies, was 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol, followed in turn by 1-*p*-aminophenyl-2-amino-1,3-propanediol, *p*-aminobenzaldehyde, *p*-aminobenzoic acid, and polymers. Thus, the major pathways would logically be *C* or *E*. The production of *p*-aminobenzaldehyde by route *C* must proceed through a series of intermediates similar to those postulated in pathway *A*. The occurrence of an arylamine derivative, similar to α -dichloroacetamido- β -hydroxy-*p*-nitropropionophenone, has not been detected with certainty as it has not been possible to obtain sufficient quantities of this compound for chemical studies necessary to elucidate its structure.

The production of primary arylamine derivatives must in all probability proceed through a series of intermediate steps from the nitro compounds. These intermediate compounds are probably very similar to those observed by Bray *et al.* (2) in the reduction of aryl nitro compounds by tissue. Using their procedures, it has been possible to detect compounds which can be diazotized and coupled after acetylation. Bray postulated that such compounds must be intermediates in the reduction of nitro compounds to arylamine derivatives. In no case did these compounds represent more than 2% of the total diazotizable compounds present in the bacterial suspensions.

The assumption that *p*-aminobenzaldehyde must be derived largely from 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol through pathway *C* rather than from 1-*p*-aminophenyl-2-amino-1,3-propanediol through pathway *E* is supported by the results obtained from studies on the utilization of these compounds as sources of *p*-aminobenzoic acid for microorganisms. It was found that 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol was a much better source of *p*-aminobenzoic acid than was 1-*p*-aminophenyl-2-amino-1,3-propanediol. If 1-*p*-aminophenyl-2-amino-1,3-propanediol were an intermediate compound in the production of *p*-aminobenzoic acid and *p*-aminobenzaldehyde from chloromycetin through pathway *E*, it should have been more active than 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol in this regard.

It is very doubtful that the conversion of chloromycetin to arylamine derivatives such as *p*-aminobenzaldehyde or *p*-aminobenzoic acid, which might be utilized by the organism is of any great significance in the decomposition of the drug by *Escherichia coli*. Once the arylamine deriv-

atives are formed, there appears to be no significant utilization of the products. The arylamine concentrations of actively growing broth cultures did not change appreciably in 3 weeks.

With *Proteus vulgaris* and *Bacillus subtilis*, decomposition of chloromycetin does not appear to proceed through one major pathway. Both organisms appear to use several pathways simultaneously to degrade the drug. These organisms produce rather large quantities of arylamine derivatives together with large quantities of relatively inactive nitro compounds. In 24 hr. a suspension of *Proteus vulgaris* converted 30–35% of the antibiotic into arylamine compounds, while *Bacillus subtilis* converted about 25–35%. *Proteus vulgaris* is, however, more effective than *Bacillus subtilis* in converting the drug into relatively inactive nitro compounds with a resulting loss in antibiotic potency of the solution. A suspension of *Proteus vulgaris* containing 100 $\mu\text{g.}/\text{ml.}$ of the antibiotic will produce for all practical purposes a complete loss in antibiotic activity within 24 hr., while a suspension of *Bacillus subtilis* under the same conditions will inactivate only 50–60% of the drug.

Both *Bacillus subtilis* and *Proteus vulgaris* produce considerable quantities of chloramphenicolase (13), the enzyme responsible for the hydrolysis of the amide linkage of chloromycetin. This results in large quantities of 1-*p*-nitrophenyl-2-amino-1,3-propanediol being produced which can in turn be rapidly converted into 1-*p*-aminophenyl-2-amino-1,3-propanediol. The rate of conversion of 1-*p*-nitrophenyl-2-amino-1,3-propanediol to 1-*p*-aminophenyl-2-amino-1,3-propanediol by the test organisms appears to be approximately twice as rapid as the rate of conversion of chloromycetin to 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol.

From the results obtained, it would appear that the major pathways employed by both *Proteus vulgaris* and *Bacillus subtilis* for the decomposition of chloromycetin are *A*, *B*, *D* and *E* with the step (*B*–*D*) being the predominant reaction.

It is interesting to note in connection with the bacterial decomposition of chloromycetin, the number of products which are formed that could be utilized by microorganisms. For example, *p*-aminobenzaldehyde, *p*-nitrobenzaldehyde, and *p*-nitrobenzoic acid, in addition to *p*-aminobenzoic acid itself, are known to exhibit aminobenzoic acid "growth-factor" activity (8). Some of the other compounds such as 1-*p*-aminophenyl-2-dichloroacetamido-1,3-propanediol and 1-*p*-aminophenyl 2-amino-1,3-propanediol have also been shown to exhibit

p-aminobenzoic acid "growth-factor" activity in preliminary tests. Ethanolamine in trace amounts appears to be utilized by the test organism and causes also a general stimulation of growth.

SUMMARY

Studies on the action of *Escherichia coli*, *Bacillus mycoides*, *Bacillus subtilis*, and *Proteus vulgaris* on chloromycetin have revealed that each of these organisms can degrade the drug by hydrolyzing the amide linkage, reducing the nitro group, oxidizing the secondary hydroxyl group, and bringing about a cleavage of the molecule between the first and second carbons of the propanediol portion of the molecule. By employing each of these reactions individually or in combination, at least 18 decomposition products can be formed which can be isolated and identified.

Five pathways have been postulated by means of which the drug can be degraded. Each of the organisms tested appears to be able to use all of the pathways, but each appears to have a preference as to which pathway or pathways it will employ. *Bacillus mycoides* appears to produce largely inactive nitro compounds from chloromycetin while *Escherichia coli* favors the production of inactive arylamine compounds. Both *Bacillus subtilis* and *Proteus vulgaris* produce large quantities of both inactive nitro and arylamine compounds. *Bacillus subtilis* produced about equal quantities of the two types of compounds while *Proteus vulgaris* produced about twice the quantity of inactive nitro compounds as arylamine compounds.

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The Occurrence of Vitamin B₁₂ and Other Growth Factors in Alfalfa¹

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INTRODUCTION

In a recent paper (10), alfalfa meal was reported to contain 40 parts per billion³ (p.p.b.) of vitamin B₁₂ by a microbiological assay. Since alfalfa is an important forage crop, this amount would make alfalfa a significant source of this vitamin in animal nutrition. The following report indicates that factors in alfalfa other than vitamin B₁₂ are measured in the microbiological assay with *Lactobacillus leichmannii*.

EXPERIMENTAL

A tube assay with *L. leichmannii* (A.T.C.C. 4797) was used. This method is a modification of the method of Skeggs *et al.* (12), involving the use of a basal medium (double strength) containing 0.2% of ascorbic acid (13) and increased concentrations of uracil (60 mg./l.), and guanine (75 mg./l.), and adjusted initially to pH 5.7.

Extraction of the Apparent B₁₂ Activity

Two methods were used in the extraction of the apparent B₁₂ activity from alfalfa. One method involved digestion of the sample with papain and taka-diaxase. The details of this technique have been published recently (10). The second method involved the refluxing of weighed amounts of alfalfa in 500 ml. of distilled water for 30 min. In certain experiments which will be described later, this time of reflux was varied. The solution containing the water-soluble, apparent B₁₂ activity was removed from the residue by filtration, the residue was washed three times with separate portions of water, and the washings were combined with the filtrate.

¹ While this manuscript was in review, a paper substantiating some of the conclusions reported herein was presented at the 117th Meeting of the American Chemical Society in Philadelphia, April 11, 1950 (PEELER, MILLER, CARLSON, AND NORRIS, Studies on the Vitamin B₁₂ Contents of Feedstuffs and Other Materials).

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³ In American and French numeration: 10⁹.

Total Apparent Vitamin B₁₂ Activity

For assay purposes the apparent B₁₂ activity was computed with a standard curve for vitamin B₁₂. However, since work herein reported showed that only a fraction of the total response obtained with *L. leichmannii* was caused by B₁₂, the apparent B₁₂ activity, computed did not follow the B₁₂ curve and varied with the size of the sample.

Fresh alfalfa was found to contain an apparent vitamin B₁₂ activity of 50–62 p.p.b. the value obtained being dependent on the size of the sample.

When dehydrated alfalfa meal was assayed with hot water used to extract the activity, a value one-fifth of that of the fresh alfalfa was obtained. Increasing the time of extraction from 15 min. to 2 hr. did not liberate additional quantities of apparent B₁₂ activity. With hot water, the pH of the extracting solution was found to be 5.9. Extraction of the meal with a hot alkali solution adjusted to pII 9.3 did not liberate any additional activity. When the pII of the extracting solution was adjusted to 3.0 or less, the apparent B₁₂ activity extracted was reduced by about 40%. This would indicate the presence of an acid-labile factor in addition to vitamin B₁₂.

When enzymic digestion was employed to liberate the apparent B₁₂ activity, considerably higher values were obtained. The value of 38 p.p.b. obtained with a 2-g. sample of good-quality meal was in close agreement with a previously reported value of 40 p.p.b. for dehydrated meal (10). Apparent vitamin B₁₂ values ranging from 15–45 p.p.b. were obtained for the same alfalfa meal when sample size was varied from 20 g. to 1 g., respectively. For these assays the size of the sample was the only variable; the quantity of reagents and technique of extraction were the same.

Chick Assay for Growth Factor

Dr. J. McGinnis of Washington State Agricultural Experiment Station performed the chick assays reported in this paper. White leghorn chicks were fed an animal-protein-factor-deficient diet for 3 days from hatching and distributed on the basis of weight into experimental groups of 12 chicks each. Each experimental diet was fed to duplicate groups. The technique and basal diet employed have been reported earlier (8).

The chicks, which were fed the alfalfa concentrate at a level of 15% of the ration, would have been supplied a diet containing about 7 p.p.b. of B₁₂ if all the apparent B₁₂ activity had been due to B₁₂. This level of B₁₂ in the ration should produce good growth (9). The lack of significant response in the chick test indicated that other factors than vita-

TABLE I

Comparison of an Alfalfa Extract With a Vitamin B₁₂ Concentrate on Chick Growth

| Supplement to basal diet | Mortality | Average weight ^a |
|--------------------------------------|-----------|-----------------------------|
| | No. died | (3 weeks) g. |
| None | 7, 5 | 73, 87 |
| Alcohol-soluble liver fraction, 0.2% | 0, 4 | 170, 168 |
| Alfalfa concentrate, 5 % | 6, 5 | 109, 97 |
| Alfalfa concentrate, 10% | 5, 4 | 110, 90 |
| Alfalfa concentrate, 15% | 7, 1 | 85, 113 |

^a Duplicate groups of 12 chicks per pen, initially.

min B₁₂ were responsible for most of the growth obtained in the assay with *L. leichmannii*, and that these factors do not cause a growth response in the chick.

Correction for Effect of Desoxyribosides

It has been reported that at least five of the naturally occurring desoxyribosides produce a microbiological response in the vitamin B₁₂ assay (14). At least three methods have been proposed to distinguish between vitamin B₁₂ and the desoxyribosides. These include alkali digestion to destroy B₁₂ (5), abnormal salt concentration in media to eliminate desoxyriboside response (3), and separation of the growth factors by paper-partition chromatography (14).

Alkali Digestion

Alkali digestion is accomplished by steaming in 0.2 *N* NaOH for 30 min. (5). Since vitamin B₁₂ is destroyed by alkali, the activity remaining after alkaline digestion is a measure of the desoxyriboside content of the sample.

When this method was applied to various alfalfa preparations the apparent B₁₂ activity for *L. leichmannii* was often higher than that of the original extract. This seems to indicate either that the activity was largely due to factors other than vitamin B₁₂ or that additional desoxyribosides were liberated during alkaline digestion. Alkali digestion may also be acting to destroy a growth inhibitor for *L. leichmannii* present in the alfalfa extract.

Salt Media

A second method recently reported for eliminating the growth response of the test culture to desoxyribonucleic acid or to its constituent

nucleosides involved the use of abnormal salt concentrations (3). According to this method the organism used in the assay does not respond to the desoxyribosides in the presence of 2% salt, whereas the response to vitamin B₁₂ is unimpaired in the presence of the salt.

Although the method was developed for use with *Lactobacillus lactis* Doinei, we attempted to apply it with *L. leichmannii*. When pure solutions of vitamin B₁₂ at a concentration of 50 mμg/ml were assayed by the cup method (3), it was found that, although excellent growth was obtained on the regular medium, very little growth was obtained on the 2% salt medium. Good response was obtained from the alfalfa extracts in both media, although the zones of growth on the salt medium were somewhat smaller.

When pure solutions of vitamin B₁ were assayed by the tube method in the presence of 2% salt, no response was obtained at low levels of the vitamin.

When higher levels of the vitamin were employed *L. leichmannii* was able to utilize it in the presence of the salt to a considerably lesser extent than on the normal medium. Thus the response of *L. leichmannii* appears to be quite different from that of *L. lactis* at least at low levels of vitamin B₁. When alfalfa was assayed in the salt medium by the tube assay, only a small reduction in growth resulted (Table II).

When known amounts of vitamin B₁₂ were added to alfalfa, the organisms again did not respond to the added B₁, (cf. samples 3 and 6).

TABLE II

Effect of 2% Sodium Chloride in Assay Media on Apparent Vitamin B₁₂ Assay

| Sample | Apparent activity calculated as crystalline vitamin B ₁ (mμg) | | | | | |
|--------|--|-----------|--------------|-------|----------------|-------------|
| | Quantities added in test solution | | | | Found by assay | |
| | Vitamin B ₁ | Thymidine | Alf. extract | Total | Regular media | Salt medium |
| | mμg | mμg | mμg | mμg | mμg | mμg |
| 1 | 0.11 | 0 | 0 | 0.11 | 0.11 | 0 |
| 2 | 0 | 0.08 | 0 | 0.08 | 0.08 | 0.02 |
| 3 | 0 | 0 | 0.12 | 0.12 | 0.12 | 0.08 |
| 4 | 0.11 | 0.08 | 0 | 0.19 | 0.18 | 0.03 |
| 5 | 0.11 | 0.08 | 0.12 | 0.31 | 0.24 | 0.14 |
| 6 | 0.11 | 0 | 0.12 | 0.23 | 0.21 | 0.09 |
| 7 | 0 | 0.08 | 0.12 | 0.20 | 0.17 | 0.12 |

Table II). Unfortunately, 2% salt partially inhibited the response to thymidine also (sample 2, Table II) so that this technique could not be used for determining the ratio of vitamin B₁₂ to thymidine in the alfalfa. However, it seems that any reduction in activity from the regular to salt media represents all the B₁₂ and a portion, at least, of the thymidine present. Conversely, any activity still present in the salt media cannot be due to vitamin B₁₂. Accordingly, these experiments with salt media would indicate that at least 66% of the apparent vitamin B₁₂ activity is not due to vitamin B₁₂ in alfalfa.

Paper Chromatography for Separation of Growth Factors

Paper-partition chromatography was employed in order to obtain further insight into the nature of the growth-promoting factor in alfalfa. The general method involving the use of paper chromatography in the analysis of vitamin B₁₂ and related bacterial growth factors has been described (11,15,16). In the present study, solutions being examined were spread on Whatman No. 1 paper strips, in bands 20 cm. in length (16). The chromatograms were developed overnight at room tempera-

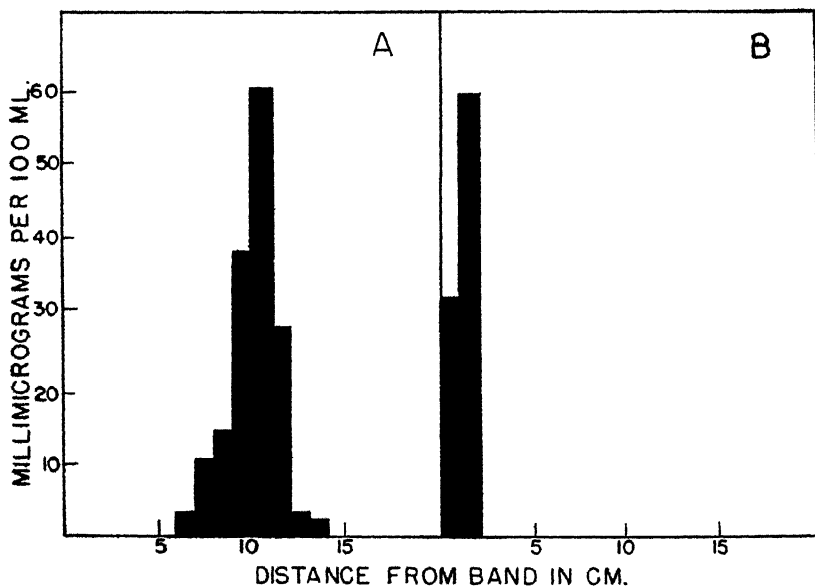


Fig. 1. Paper chromatogram of thymidine (A) and of crystalline vitamin B₁₂ (B).

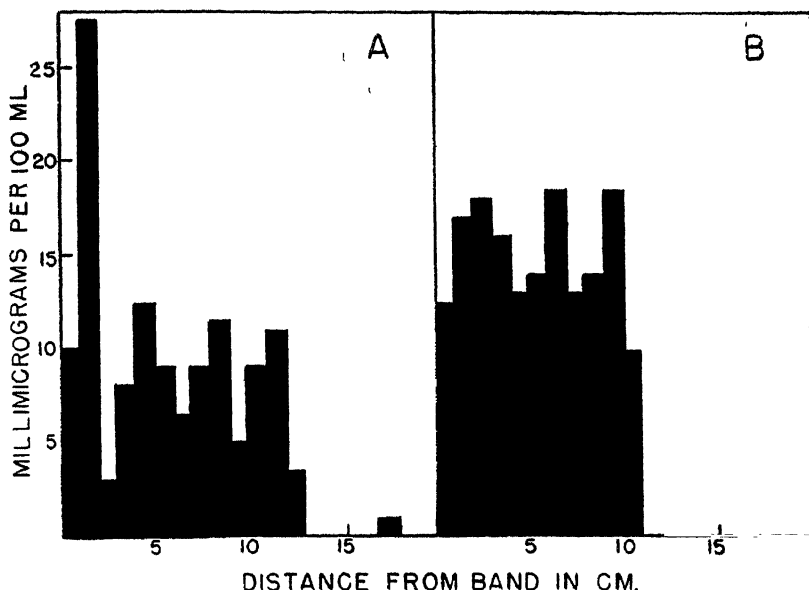


FIG. 2. Paper chromatogram of alfalfa extract before (A) and after alkaline digestion (B).

ture by upward development with wet butanol. The solvent was allowed to travel 20 cm. The strips were then dried before a fan at room temperature and cut crosswise into twenty 1-cm. sections. Each of the strips was placed in a separate test tube to which 8 ml. of regular assay medium was added, the tubes were inoculated with *L. leichmannii*, incubated, and subjected to the regular tube assay.

Histograms for vitamin B₁₂ and for thymidine are presented for comparison in Fig. 1. It was found that the ratio of thymidine to vitamin B₁₂ for comparable growth response was about 15,000 to 1.

The alfalfa histogram (Fig. 2A) demonstrates clearly that the growth-promoting activity is due to a number of factors. At least four peaks are present, two of which correspond closely with those for vitamin B₁₂ and for thymidine. The other peaks are in positions intermediate between these two. The zone corresponding to the position of thymidine in the alfalfa chromatogram contains about 25% of the total activity.

Alkaline digestion of an alfalfa extract to destroy the vitamin B₁₂ resulted in only partial reduction of the peak in the region correspond-

ing to the vitamin B₁₂ activity (Fig. 2B). When known amounts of vitamin B₁₂ were added to an alfalfa extract and the mixture chromatographed, the B₁₂ peak became superimposed on the first peak of the alfalfa extract (Fig. 3A). After alkaline digestion, almost all of the activity due to the added vitamin B₁₂ was lost, but a sizable peak still remained in this region (Fig. 3B). This is analogous to the situation after alkaline digestion of an alfalfa extract alone (Fig. 2B) and indicates that a large amount of the activity that occurs in the zone normally associ-

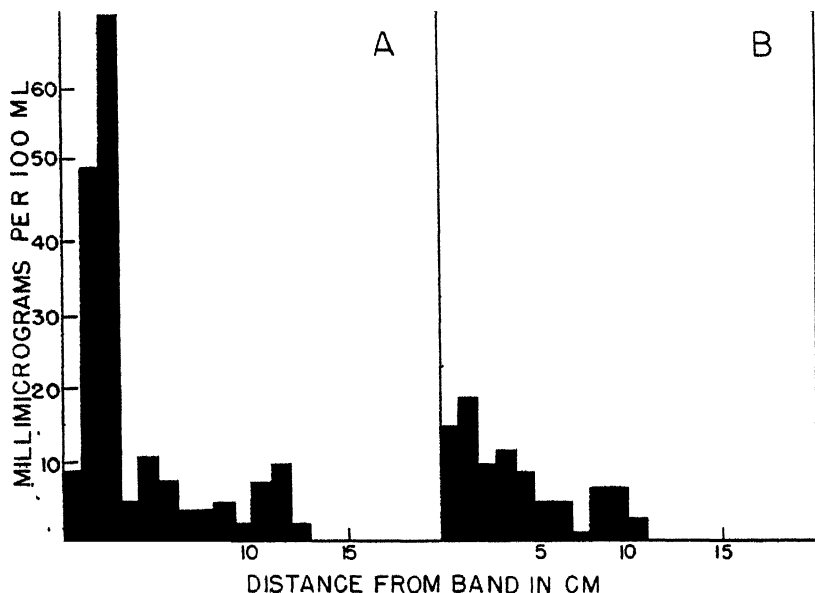


FIG. 3. Paper chromatogram of alfalfa extract plus added vitamin B₁₂ before (A) and after alkaline digestion (B).

ated with vitamin B₁₂ must be due to another factor. Whether this factor is one of the naturally occurring desoxyribosides has not been ascertained in this investigation. However, it appears from these chromatographic studies that more than 85% of the apparent vitamin B₁₂ activity of the alfalfa is due to factors other than vitamin B₁₂.

Pure vitamin B₁₂ can be transferred quantitatively to butanol from water by saturation with ammonium sulfate. When this technique was applied to an extract of alfalfa about 25% of the activity was trans-

ferred to the butanol. Paper-partition chromatography showed that the butanol extract was rich in the faster-migrating factor and the total apparent B₁₂ activity was not reduced by alkali hydrolysis, indicating that little, if any, activity in the butanol extract was due to vitamin B₁₂.

Concentration of the Growth-Promoting Factor

An attempt was made to concentrate the growth-promoting activity in order to determine some of its properties. Various fractions prepared from fresh alfalfa in connection with another project (1) were surveyed for apparent vitamin B₁₂ content. It was found that the hot-water extract of alfalfa bagasse, which resulted from the expression of the whole juice from fresh alfalfa, contained the highest apparent B₁₂ content of the various fractions tested. This fraction was selected for further concentration studies.

In a recent publication, Emerson *et al.* (2) gave the details of a technique employed for the concentration of vitamin B₁₂ from fish solubles and reported that the method had been successfully applied to alfalfa. The over-all efficiency of this procedure was very low in this laboratory, since sharp separations were not accomplished. Large losses of the factor resulted in each operation.

Properties of the Growth Factor

The one or more factors were found to be dialyzable through Visking cellophane, and only partially precipitated by 70% alcohol, ammonium sulfate, or lead acetate. Although a portion of the activity was precipitated by adjustment to either pH 3 or 1, other material was precipitated also, so that no concentration of the activity resulted either in the precipitate or the filtrate. The most promising techniques devised for concentration were acetone precipitation, chromatography on silica gel, and adsorption and elution from charcoal.

Eighty Per Cent Acetone Concentration

When acetone was added to the water extract to give a final acetone concentration of 80%, a large amount of sediment was deposited, resulting in a large increase in the concentration of the factor in the acetone-soluble fraction, when calculated to a moisture-free basis.

In a typical experiment, 99 g. of a hot water extract of bagasse containing 36.6% H₂O was diluted to a volume of 200 ml. with water and slowly added to 600 ml. of

TABLE III

Effectiveness of 80% Acetone for Concentrating L. leichmannii Activity from Alfalfa

| Material | Weight | Water | Apparent vitamin B ₁₂ activity (moisture-free basis) | Activity in fraction |
|-------------------------|--------|-------|---|----------------------|
| | g. | % | p.p.b. | % |
| Original extract | 99.0 | 36.6 | 225 | |
| Filtrate from 1st pptn. | 55.5 | 56.0 | 900 | 79 |
| Filtrate from 2nd pptn. | 50.5 | 79.4 | 440 | 14 |
| Filtrate from 3rd pptn. | 108.2 | 89.1 | 95 | 4 |
| Precipitate | 242 | 83.8 | 21 | 3 |

acetone. The mixture was stored overnight at -23°C . The supernatant extract was decanted from the precipitate, was taken up in water and reprecipitated twice more with 80% acetone. The three extracts were analyzed separately. The results showed that about a four-fold concentration of the apparent B₁₂ activity resulted (Table III).

Chromatography on Silica Gel

A chromatographic column 2.5 cm. in diameter was packed to a height of 40 cm. with silica gel. Ten ml. of an 80% acetone-soluble alfalfa extract containing 1.61 g. solids and 467 p.p.b. apparent B₁₂ activity was added to the column. The column

TABLE IV

Concentration of L. leichmannii Activity by Chromatographic Separation on Silica Gel

| Fraction | Volume collected | Fraction of added solids | Fraction of added apparent B ₁₂ activity | Apparent B ₁₂ concentration |
|----------|------------------|--------------------------|---|--|
| | ml. | % | % | p.p.b. |
| 1 | 81 | 55 | 30 | 476 |
| 2 | 75 | 14 | 15 | 968 |
| 3 | 75 | 9 | 14 | 1300 |
| 4 | 75 | 7 | 13 | 1785 |
| 5 | 75 | 4 | 13 | 3000 |

was developed with distilled water and five separate fractions were collected. One fraction obtained by this procedure assayed 3000 p.p.b. total apparent B₁₂ activity (Table IV).

The above procedures did not preferentially concentrate either the pure B₁₂ or the non-B₁₂ activity.

DISCUSSION

Several reports based on chick (6) and rat (7,17) assays have recently stated that alfalfa contains no significant quantities of vitamin B₁₂ or related growth factors, while others have reported it to contain measurable quantities of vitamin B₁₂, based on rat (4), chick (2), or microbiological (10) assay.

The microbiological technique will lead to misleading results with alfalfa, since there is a high proportion of other factors present to which *L. leichmannii* responds. It appears likely that these factors may consist of the naturally occurring desoxyribosides which have been shown to respond to the B₁₂ assay. Of the total apparent B₁₂ activity in alfalfa, not more than 15% appears to be actually due to vitamin B₁₂.

Chick-growth assays with our alfalfa fractions have shown little if any response.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Mrs. P. A. Thompson in the large number of assays required. We are also grateful to Dr. William Shive for the thymidine used.

SUMMARY

Microbiological assays for vitamin B₁₂ in alfalfa using *L. leichmannii* as the test organism yielded total apparent vitamin B₁₂ values ranging from 50 to 62 parts per billion (p.p.b.) for fresh alfalfa and from 12 to 45 p.p.b. for dehydrated alfalfa meal. The values obtained varied with sample size, the smaller the sample, the higher the apparent B₁₂ activity.

Employment of differential assay techniques such as alkali digestion to destroy B₁₂, inclusion of salt in the media which inhibits response to B₁₂, and paper-partition chromatography have indicated that more than 85% of the total apparent vitamin B₁₂ activity is due to factors other than B₁₂. Chick assays indicate that the factors responsible for the apparent B₁₂ activity in microbiological assays do not replace vitamin B₁₂ for chick growth.

Several techniques, including precipitation of impurities with acetone, adsorption of activity on charcoal, and chromatography on silica gel have proved successful in concentrating the factors responsible for the apparent B₁₂ activity of alfalfa, but did not preferentially concentrate the pure B₁₂.

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The *in vivo* Inhibition of Succinic Dehydrogenase by Selenium and Its Release by Arsenic¹

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INTRODUCTION

Several investigators (1-11) have reported the inhibition of succinic oxidase activity of animal tissues by selenium compounds *in vitro*. Also, work at two laboratories (4,11) has shown that the succinic dehydrogenase is the part of the succinic oxidase system that is inhibited by selenium.

In 1938, Moxon (12) reported the alleviation of selenium poisoning in albino rats by arsenite. Since then, arsenic has been used to counteract selenium poisoning in various farm and laboratory animals (13-21). Relatively little information is available concerning the mode of action of this selenium-arsenic antagonism.

The present report deals with the effect of dietary casein levels on rat liver succinic dehydrogenase activity and the *in vivo* inhibition of the enzyme by selenite together with the release of the inhibition by arsenite.

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² This paper represents a portion of a thesis submitted by Harlan L. Klug to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

EXPERIMENTAL AND RESULTS

Effects of Casein Levels on Liver Succinic Oxidase

Since protein intake affects the concentration of liver enzymes (22-24), it was decided to determine the minimum protein level that would maintain a nearly constant activity of succinic dehydrogenase in rat livers. Such a diet would be expected to be the most suitable for demonstrating alterations in liver succinic dehydrogenase by selenium compounds. The identification of the groups and the composition of the diets are shown in Table I. An extra group (VII) was introduced later in the experiment in order to test for a possible riboflavin deficiency in the 20% casein diet. Each animal in this group received a daily supplement of 100 μ g. of riboflavin.

Male albino rats weighing approximately 110 g./animal were used in groups of four animals each. The animals were kept in individual cages and were fed and watered *ad lib*. Two rats from each group were sacri-

TABLE I
Percentage Composition of the Diets and Group Identification
Each animal in the following groups (except VI) received
100 mg. of dried brewer's yeast^a daily.

| Group number | I | II | III | IV | V | VI | VII |
|--|------|------|------|------|------|---------------------------------|------------------------------|
| Casein ^a | 0.0 | 5.0 | 10.0 | 20.0 | 40.0 | Check diet | 20.0 |
| Starch ^b | 83.5 | 78.5 | 73.5 | 63.5 | 43.5 | Purina dog chow ^d | 63.5 |
| Fat (11.25% lard and 0.75% A and D feeding oil ^c) | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 | | 12.0 |
| Salt mix (25) | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | | 4.5 |
| Riboflavin (Merek) | | | | | | | 100 μ g. daily/animal |

^a B-3-F casein (not vitamin free) prepared by the Casein Company of America, New York, N. Y.

^b Argo corn starch manufactured by the Corn Products Refining Company of Argo, Illinois.

^c A and D feeding oil (3000 international units of Vitamin A and 400 I. U. of Vitamin D/g.).

^d Manufactured by Ralston Purina Company, St. Louis, Missouri.

^e Type 50-B produced by Standard Brands Incorporated, New York, N. Y.

ficed on the second day and fourth or fifth day of the experiment, and the livers were assayed for succinic dehydrogenase by the method of Schneider and Potter (26).

The rats receiving the 10% casein diet (Table I) maintained their initial body weights. The animals fed the higher casein levels showed steady weight gains, while those receiving no casein showed weight losses. It was observed that the 20% casein diet was eaten more readily than any of the other experimental diets.

TABLE II

The Effect of Casein Levels on Liver Succinic Oxidase
Each value represents an average of duplicate flasks per animal.
The data are reported in terms of Q_{O_2} succinate.

| Days on experiment | Group number I Per cent casein: 0.0 | II 5.0 | III 10.0 | IV 20.0 | V 40.0 | VI Check | VII 20.0 + riboflavin |
|--------------------|--|--------------|--------------|--------------|--------------|--------------|-----------------------------|
| 0 | | | | | | 82.0 81.0 | |
| 2 | 47.0 51.2 | 54.1 52.8 | 52.0 42.8 | 65.3 73.1 | 78.4 85.5 | 89.0 83.0 | 56.1 63.5 |
| 3 | | | | | | | 56.0 |
| 4 | | | | | | | 54.0 |
| 5 | 66.5 46.4 | 53.4 49.3 | 52.0 52.1 | 56.0 53.0 | 71.4 81.4 | 89.1 89.2 | |

Results of the liver succinic dehydrogenase assays are shown in Table II. Of the casein-fed groups, animals receiving the 40% diet maintained the highest enzymatic activity. A decrease in activity of approximately 30% resulted in feeding diets containing less than 20% casein. It was concluded that the 40% casein diet was an appropriate basal synthetic diet for testing the effects of selenium, arsenic, and selenium plus arsenic on succinic dehydrogenase. It is possible that a 30% casein diet would have been satisfactory but this was not tested.

*The Effects of Selenium, Arsenic, and Selenium Plus
Arsenic on Liver Succinic Dehydrogenase*

Rats weighing approximately 110 g./animal were used in five experimental groups of 10 animals each. These groups received the following diets:

| Group number | Diet |
|--------------|---|
| I | Basal diet |
| II | Basal diet + selenium (35 p.p.m.) as Na_2SeO_3 . |
| III | Basal diet + arsenic (17.5 p.p.m.) as NaAsO_2 in the drinking water. |
| IV | Basal diet + selenium (35 p.p.m.) + arsenic (17.5 p.p.m.) |
| V | Purina dog chow: check group. |

The basal diet had the composition shown under group V, Table I. The sodium selenite was incorporated in the salt mixture and then mixed thoroughly with the rest of the diet. The arsenic was added to the drinking water (distilled) and a fresh solution was prepared every 5 days. The selenium content of the diet and the arsenic content of the water were checked by analyses of samples taken at intervals throughout the experiment.

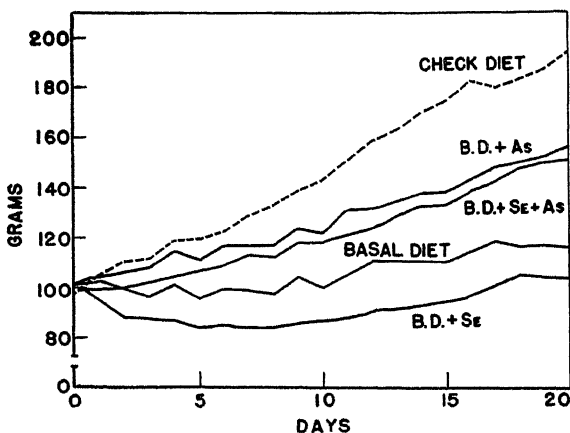


FIG. 1. The daily average weights of rats receiving selenium, arsenic, and selenium plus arsenic. B. D. designates basal diet.

The animals were pair fed, that is individual animals in groups I and III were paired to the animals of groups II and IV, respectively. This method of feeding corrected for the decreased food intakes of the selenium-fed animals (27,28). The animals of group V were fed *ad lib*. The daily average weights of the groups are shown in Fig. 1.

The enzyme assays showed decreased amounts of succinic dehydrogenase in the livers of the animals receiving diets containing selenium

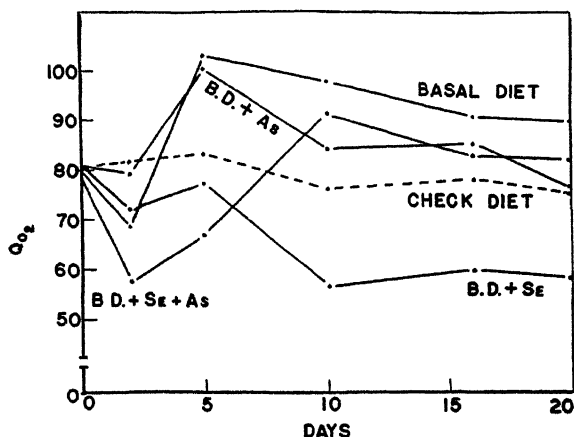


FIG. 2. The effects of selenium, arsenic, and selenium plus arsenic on liver Q_{O_2} succinate. Each value represents an average of duplicate assays per animal for two animals.

(Fig. 2). Especially interesting was the effect of arsenic in facilitating the return of Q_{O_2} succinate to normal levels after the ninth day. Duplication of this experiment confirmed these results.

DISCUSSION

It is clear that selenium decreases the liver Q_{O_2} succinate values. Although the lowered enzyme concentration may be due to an interference with its synthesis, it is more likely that the concentration of active enzyme is being decreased by an inactivation that outpaces the synthetic process. Current experiments, designed to investigate the effect of selenium on protein absorption and utilization, have established the fact that fasting rats have greater than normal succinic dehydrogenase values for liver tissue.

The fact that selenium reacts readily with mercapto groups of organic compounds *in vitro* plus the fact that succinic dehydrogenase is a mercapto enzyme (29-34) suggests that selenium is reacting with the mercapto groups and thus inactivating the enzyme. Whether the inactivation is brought about by oxidation or by a mercaptide formation (33-35) is not known.

The enzyme data for the animals in group IV (basal diet + Se + As) are especially interesting in terms of the selenium-arsenic antagonism.

Both of the selenium-containing diets caused decreased QO_2 values during the first 7 or 8 days of the experiment. After this initial period the succinic dehydrogenase concentration returned to a normal value in the group getting the selenium plus arsenic.

Whether succinic dehydrogenase is the most sensitive enzyme system to selenium toxicity remains to be determined; however, pyruvic oxidase and triose phosphate dehydrogenase are usually considered to be more sensitive to mercapto-inhibiting agents than succinic dehydrogenase (34).

SUMMARY

A synthetic diet, which maintains a relatively high rat liver succinioxidase level, has been established.

It has been shown that selenium lowers the liver succinic dehydrogenase levels of rats fed this diet. The inclusion of arsenic in the selenium diet restores the enzyme values to normal after a relatively short period of depression.

The results suggest that the toxic action of selenium in the animal body is due, in part, to the inactivation of succinic dehydrogenase.

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The Specificity of Pectinesterases from Several Sources with Some Notes on Purification of Orange Pectinesterase

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INTRODUCTION

The characteristics of pectinesterase (PE), although known for more than 100 years, have received increased study during the past few years. However, studies have not included determination of the specificity of PE but rather have described the interrelation of cations and pH on PE activity (1-6) and have described the extraction characteristics of pectinesterases from several sources (3,4,7,8).

The impression has developed without very good evidence that pectinesterase is not a specific enzyme (5,9). This idea appears to be based on the observations of Ehrlich and Guttman (10), who used lucerne PE preparations to hydrolyze the ester linkage of α -methyl-D-galacturonic acid methyl ester; and of Neuberg and Ostendorf (11), who used the calcium salt of methyl-D-tartaric acid as model substrate in their study of tobacco PE. This substrate was used also in a study of the PE content of certain plant-pathogenic fungi and bacteria (12).

Obviously another enzyme, or other enzymes, occurring in crude pectinesterase preparations might have hydrolyzed these nonpectin substrates. This is the case with orange flavedo pectinesterase, since purified PE hydrolyzes esters of polygalacturonic acid only, whereas crude enzyme preparations hydrolyzed a number of esters [see abstract report (13)]. Jansen, Jang, and MacDonnell (14) have recently described some of the characteristics of an esterase of citrus fruit that

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does not act on citrus pectin and have named it acetylesterase, since it hydrolyzed most rapidly esters of acetic acid.

Literature that suggests that pectinesterase is highly specific includes a report by Kertesz (15) that a tomato pectinesterase preparation did not hydrolyze α -methyl-D-galacturonide methyl ester; a report by Line-weaver and Ballou (1) that alfalfa (lucerne) pectinesterase preparations hydrolyze neither this compound nor the methyl ester of galacturonic acid; and, more recently, a report by Deuel (16) that neither citrus, alfalfa, tomato, nor *Carica papaya* PE was able to hydrolyze the glycol or glycerol ester of pectic acid.

This paper presents a study of the specificity of the pectinesterases from three plant sources, alfalfa, orange, and tomato, and from one fungal source.² The results revealed that 28 esters were not hydrolyzed by crude preparations from any of the sources. Eleven esters were hydrolyzed slightly by one or more of the preparations. Sixteen esters besides pectin (and the methyl and ethyl esters of polygalacturonic acid) were hydrolyzed significantly by all of the enzyme preparations, but the hydrolysis was not related to the PE content of the preparations. None of these compounds was hydrolyzed at a significant rate by purified orange PE. In addition, the method of purification of orange PE is given briefly and limited consideration is given to the character of some of the nonpectin esterases present in the enzyme sources.

MATERIALS AND METHODS

Alfalfa (lucerne) PE was prepared from freshly cut alfalfa. The alfalfa was ground in a meat grinder to a slurry which was mixed with an equal volume of 0.5 *M* sodium acetate. After standing 1 hr. the solid residue was removed by squeezing through cheesecloth. The extract was cooled to 0°C., adjusted to pH 4.7, and stored at 5°C. for 48 hr. The enzyme was precipitated from the filtrate at 0.7 saturation with $(\text{NH}_4)_2\text{SO}_4$ at pII 6. The precipitate was collected with the aid of diatomaceous earth (Celite), dried over P_2O_5 , and stored at 5°C.

Tomato PE was prepared from dry tomato solids from which the "serum" had been removed by squeezing through cheesecloth before drying. The enzyme was extracted in slightly alkaline salt solution and precipitated at pH 7 by 0.7 saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected with the aid of Celite, dried over P_2O_5 , and stored at 5°C.

The fungal PE used in this study was Pectinol 100D² unpurified.

Crude orange flavedo PE was prepared from a slightly alkaline salt extract of dry

² Fungal pectinesterase was a commercial preparation made by The Rohm and Haas Co. and designated by them as 100D. (Mention of trade names does not imply recommendation of product over others not named.)

flavado that had been passed through a meat grinder. The filtered extract was cooled to 5°C. and precipitated with 2 vol. of acetone previously cooled to -20°C. Diatomaceous earth (Celite) was added and the precipitate collected by filtration in a 0°C. room. The solvent was removed by vacuum to prevent inactivation that occurs if the temperature of the precipitate rises appreciably in the presence of acetone. The dried preparation was stored at 5°C.

Purified flavado PE was obtained by a fractionation and adsorption technique. The following procedure is one of several used in this laboratory to prepare highly purified samples of PE.

Orange flavado (fresh or frozen) is ground in a meat grinder to small particle size and extracted in 0.25 *M* NaCl at pH 7-8 for 1 hr. with alkali addition as required to maintain the pH in this region. The larger flavado particles are removed by screening through cheesecloth to give a cloudy extract, which is brought to 0.4 saturation with $(\text{NH}_4)_2\text{SO}_4$ while the pH is maintained above 7. The precipitate formed is removed with the aid of diatomaceous earth and discarded. The filtrate is brought to 0.8 saturation with $(\text{NH}_4)_2\text{SO}_4$ (pH maintained above 7). One to 2 g. of filter paper pulp/l. is added with stirring, and the suspension is filtered. The pulpy mass is wrapped in a filter cloth and pressed in a Carver laboratory hydraulic press until it is nearly free from residual $(\text{NH}_4)_2\text{SO}_4$ solution. The aqueous solution of the pressed cake, after removal of the paper pulp, is sufficiently salt-free to be used in the adsorption step without dialysis.³ Pectinesterase can be adsorbed on diatomaceous earth (Celite) (2,3,8) and on an artificial zeolite (Ducil). It can be adsorbed on or is inactivated by bentonite clay. The zeolite in contrast with Celite cannot be used without dialysis, because the pH becomes too low due to adsorption of cations. Celite No. 505, which was used at this step, was found to adsorb about 0.4 unit of PE/g. from the press-cake solution. A Sparkler filter press was used for adsorption and elution. The solution was passed through the bed of Celite several times. The bed was then washed with 0.025 *M* Na_2HPO_4 solution, and finally the enzyme is eluted with a solution containing 1 mole of NaCl and 0.025 mole of Na_2HPO_4 /l. The eluate, which contained varying though small quantities of acetylerase, is precipitated at 0.9 saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 7. The precipitate is taken up in a small amount of water and dialyzed. In this stage of purification the enzyme is fairly stable to dialysis. The dialyzed solution is treated with Ducil, which takes up about 0.75 unit of PE/g. The Ducil is washed and eluted in the same manner as the Celite. The eluate is exhaustively dialyzed and then freeze-dried to yield a slightly off-white powder. Table I contains illustrative data of the purification obtained by this process.

Most of the *substrates* used in this study were Eastman Kodak products of the Eastman grade. Exceptions were methyl acetate and diacetin, which were of the practical grade, and the oleates, which were of the technical grade. The following sub-

³ If the salt extract is directly precipitated at 0.8 $(\text{NH}_4)_2\text{SO}_4$ saturation, it may be dialyzed salt-free with about a 30% over-all loss of PE; however, after the 0.4 saturation fraction is removed the PE is even more rapidly inactivated by dialysis. It has been found that the 0.4-0.8 saturation fraction can be stabilized to dialysis by dialyzing against either 0.05 *M* sulfite or 0.05 *M* thioglycol. The use of sulfite, of course, does not yield a desirably low ionic strength. An important advantage of the pressing step is that small and therefore more convenient quantities of solution are obtained.

TABLE I
Partial Purification of Orange Flavedo Pectinesterase (PE)

| Fraction | PE | AE ^a | PE | PE yield |
|--|--------------|-----------------|------------------------------------|----------|
| | <i>units</i> | <i>units</i> | <i>units/ mg N^b</i> | <i>%</i> |
| 156 l. of extract from 108 lb. of flavedo ^c | 2045 | 148 | 0.016 | (100) |
| 164 l. of 0.4 saturated (NH ₄) ₂ SO ₄ supernatant | 1677 | 71 | | 82 |
| 185 l. of 0.8 saturated (NH ₄) ₂ SO ₄ supernatant (discard) | 2830 | | | |
| 11.5 l. of redissolved 0.8 saturation precipitate | 980 | 17 | | 47.8 |
| Adsorbed on Celite No. 505 (by difference) | 773 | | | 37.7 |
| Eluate: 0.025 M Na ₂ HPO ₄ (discard) | 49 | | | |
| 11 l. of eluate: 1 M NaCl made to 0.025 M Na ₂ HPO ₄ ^d | 320 | | | 15.6 |
| 800 ml. of solution of precipitate obtained at 0.9 saturation of (NH ₄) ₂ SO ₄ | 316 | 1 | | 15.4 |
| Solution after dialysis | 218 | 0.7 | | 10.6 |
| Adsorbed on Ducil | 204 | | | 10.0 |
| Eluate from Ducil | 172 | 0.06 | | 8.4 |
| Eluate after dialysis | 159 | <0.06 | 1.98 | 7.8 |
| Powder (prepared by freeze-drying); recovery, 703 mg. ^e | 129 | | 1.70 | 6.3 |

^a Determined as previously described (13).

^b Protein nitrogen determinations were made; however, the results even on the highly purified and dialyzed material differ markedly from total nitrogen.

^c Fresh frozen material stored for about 1 year. Extract contains about half the enzyme obtained from fresh raw material.

^d Recovery at this step was particularly poor in this experiment because of unavoidable delays encountered during processing.

^e Equivalent to 183 units/g. A previous preparation assaying 160 units/g. exhibited by electrophoretic study a component comprising about 60% of the total protein. This component contained the pectinesterase activity.

strates were not Eastman products: Ethyl acetate was a reagent grade chemical and the methyl gallate was prepared at this laboratory. H. S. Olcott of this laboratory supplied the methyl polyglutamate (17). Polygalacturonic acid polymethyl ester was prepared from commercial pectic acid by the method of Morell and Link (18). Polygalacturonic acid polyethyl ester was made in a similar manner except that the reaction was run at 65°C. rather than at the reflux temperature of ethanol. The polymethyl ester was completely esterified (16.3%); the polyethyl compound contained about 11.7% ethoxy group, which corresponds to about 50% esterification. The polymethyl ester of alginic acid, a polymannuronic acid, was prepared according to Jansen and Jang (19). It contained 11.7% methoxy group.

PE activities were determined by the essentially continuous titration procedure pre-

viously described (2,3). Under the standard assay conditions 1 mequiv. of COOH liberation/min. is designated as one unit of activity (2,3). The standard assay conditions employed were: 0.5% pectin, pH 7.5, and 0.15 *M* NaCl for the orange and tomato PE; 0.5% pectin, pH 5.7, and 0.15 *M* NaCl for the alfalfa PE; and 0.5% pectin, pH 4.5, and 0.05 *M* CaCl₂ for fungal PE.

Esterase activity tests on substrates other than pectin were made at $24 \pm 1^\circ\text{C}.$, and at two pH values (6.8 and 4.5) in order to minimize the possibility that failure to find hydrolysis of a substrate would be due to use of the "wrong" pH. The fungal enzyme was run only at pH 4.5, since it exhibited little or no PE activity at pH 6.8. At both pH values amounts of enzyme were used that would hydrolyze pectin under the imposed conditions at a rate of 1 mequiv./min. except where these amounts of enzyme gave such high rates of hydrolysis that they could not be measured accurately. At pH 6.8 the test mixture contained 0.1 g. of substrate (regardless of solubility), 3 ml. of 1 *M* acetate buffer, and enzyme; at pH 4.5 the test mixture contained 0.1 g. of substrate, 1 ml. of 1 *M* CaCl₂, 1 ml. of 0.1 *M* 4.5 acetate buffer, and enzyme. In both cases the mixture (20 ml. total volume) was placed in small screw-capped vials. The pH values of these weakly buffered mixtures were adjusted to the designated pH with 0.02 *M* NaOH after 0, 1, 3, 6, and 24 hr. At the end of each period the alkali required for pH adjustment was noted. Obviously, for substrates that were hydrolyzed, the pH decreased during each interval. Therefore it would be more exact to refer to activities in pH regions but we shall refer to pH 6.8 and 4.5 for convenience. To eliminate large errors due to pH change, short-time runs were made if the pH decreased more than 0.5 unit during any interval. For pH measurement and for stirring while alkali was added, the long slender electrodes (Beckman Nos. 290-X6 and 270-X5) were inserted into the vials, which were rotated individually on a small platform mounted on the shaft of a slow-speed motor. The pH values were always checked with the motor off to avoid errors due to stirring potential. For incubation the vials were placed on a slow motion rocking machine to keep incompletely soluble substrates agitated. Duplicates were run in all cases and blanks (without enzyme) were run at pH 6.8. Blanks were negative in all but a few cases. Although PE acting on pectin is not generally inhibited by preservatives, none was used in the tests to avoid the possibility that the action of PE on some other substrate might be inhibited by the preservative. In no case did rate of hydrolysis increase with time; an increase in rate would have indicated bacterial growth. Even so, the 24-hr. runs were used only to confirm negative results obtained for the shorter time intervals.

RESULTS

Since the primary object was to determine whether any esters other than pectin esters were hydrolyzed by pectinesterase, the activities of the enzymes on the various substrates are expressed in Fig. 1 as units of esterase activity per unit of pectinesterase activity. One unit of activity corresponds to the hydrolysis of 1 mequiv. of ester bonds/min. The experimental error was such that as little as 0.0001 unit of esterase activity per unit of pectinesterase activity could be determined. This

limit of activity corresponded to 0.7 ml. of 0.02 *N* NaOH in the titration setup and an average pH change of 0.15–0.2 unit in 24 hr. The plant esterases in all cases exhibited greater activity at pH 6.8 than 4.5; hence, all the data presented are from the higher pH runs, except, of course, for the fungal enzyme.

Nonhydrolyzed Esters

The following substrates were not hydrolyzed within experimental error (less than 0.0001 unit/PE unit) by any of the four crude pectinesterase samples. Methyl esters of the following acids: *p*-aminobenzoic; anthranilic; *o*-benzoylbenzoic; *o*-, *m*-, and *p*-bromobenzoic; carbonic;

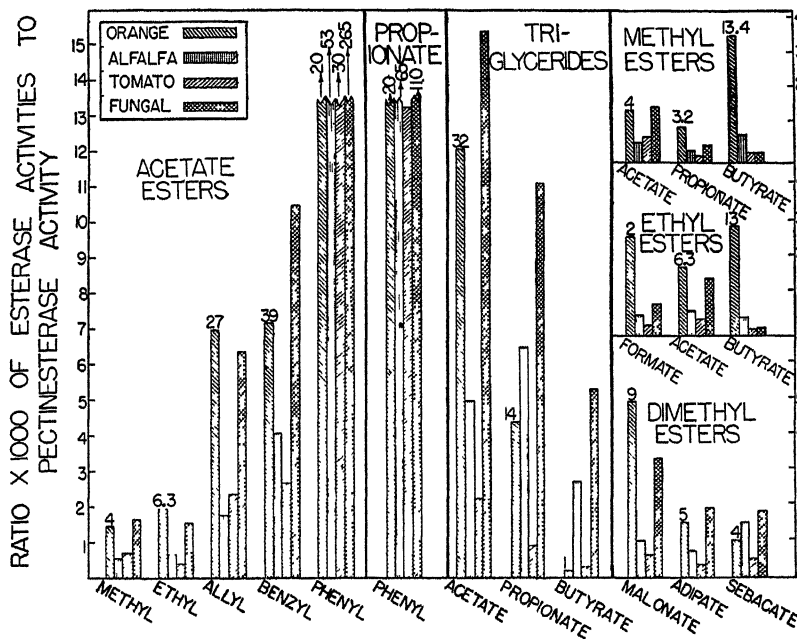


Fig. 1. Comparison of the esterase activities of orange, alfalfa, tomato, and fungal enzyme preparations compared with the pectinesterase activity of these preparations. The upright numbers above the orange figures represent the percentage of substrate hydrolyzed in 24 hr. In cases where more than 8% of the substrate was hydrolyzed in 6 hr. the figure given was calculated to a 24-hr. basis. The vertical figures above the phenyl esters refer to activities rather than per cent hydrolysis. (The calculated per cent hydrolysis for these esters is over 100% for 24 hr.)

o-chlorobenzoic; 3,5-dibromoanthranilic; *p*-hydroxybenzoic; *o*-, *m*-, and *p*-nitrobenzoic; *o*-nitrophthalic; oleic; palmitic; phthalic; polyglutamic; pyromellitic; salicylic; stearic; and hendecenoic. Also nonhydrolyzed were ethyl laurate, ethyl oleate, ethyl stearate, *N*-acetyl-*o*-aminophenol, *N*-acylethanolamine, procaine, and the methyl ester of alginic acid (polymannuronic acid).

Substrates Hydrolyzed by the Crude Enzyme Preparations

The relative activities of the four crude pectinesterase preparations acting on "hydrolyzable" substrates are reported by grouping the substrates into classes (Figs. 1 and 2).

In Fig. 1 the esterase activities per unit of PE activity for 16 substrates are seen to vary markedly from substrate to substrate and from enzyme preparation to enzyme preparation. The variation from preparation to preparation shows, as expected, that the esterase activities are either not due to PE or that the PE in various preparations differs quantitatively in specificity.

In Fig. 2 the esterase activities are compared with methyl acetate esterase activity. It is evident that a single esterase is not responsible for the various nonpectin esterase activities of the four enzyme preparations.

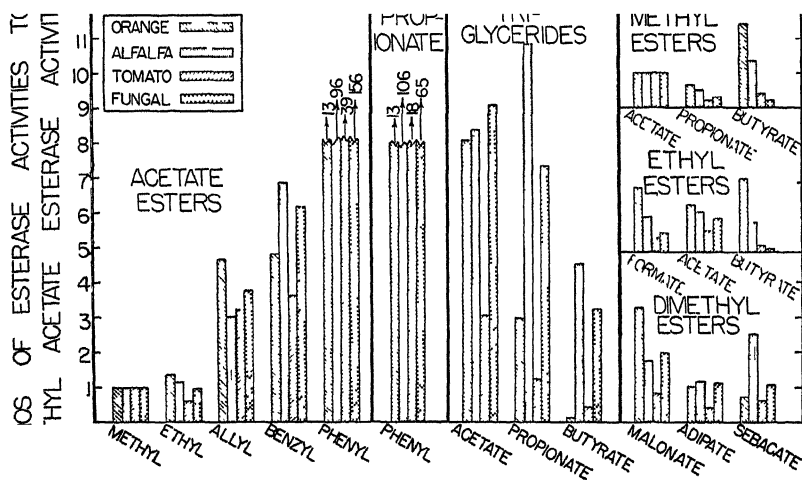


FIG. 2. Comparison of the esterase activities of orange, alfalfa, tomato, and fungal enzyme preparations with methyl acetate esterase activity of these preparations.

The following esters were hydrolyzed at a slow rate (1/1000th or less of the rate of pectin hydrolysis) by one or more of the crude enzyme preparations. These esters do not fit easily into the classes of substances reported in Figs. 1 and 2. The esterase activities per 1000 units of PE of the active crude enzyme sources are reported in parentheses for those cases where the activities were significantly greater than zero. Methyl benzoate (orange, 0.6; and fungal, 1.0), methyl cinnamate (fungal, 0.9), methyl gallate (alfalfa, 1.0), methyl phenyl acetate (orange, 2.2; and fungal, 0.5), methyl pelargonate (orange, 0.2; and alfalfa, 0.3), ethyl lactate (orange, 0.4; and alfalfa, 0.4; others not tested), ethyl tartrate (orange, 0.3), acetylcholine (orange, 0.6; alfalfa, 0.4; and fungal, 0.9) and benzyl *n*-butyrate (alfalfa, 0.9; and fungal, 0.7).

TABLE II
*Activities of Several Pectinesterase (PE) Preparations on
Methyl- and Ethylpolygalacturonate*

| Enzyme preparation | Relative activities on various substrates | | |
|--------------------|---|---------------------------------------|--------------------------------------|
| | Pectin | Methyl polygalacturonate ^a | Ethyl polygalacturonate ^b |
| Crude alfalfa PE | 100 | 58 | 3.7 |
| Crude tomato PE | 100 | 40 | 2.8 |
| Crude fungal PE | 100 | 80 | 13.1 |
| Crude orange PE | 100 | 47 | 4.0 |
| Purified orange PE | 100 | 50 | 4.1 |

^a Completely esterified.

^b Approximately 50% esterified.

Of special interest is the hydrolysis of the ethyl ester of polygalacturonic acid (Table II). The rates of hydrolysis of the ethyl ester by the four enzyme preparations are 6-16% of the rate of hydrolysis of the methyl ester. That ethyl ester rather than methyl ester bonds were being hydrolyzed follows from the fact that during the rate studies 6% of the total (free and combined) carboxyl groups in the substrate were freed whereas less than 2% of them could have been esterified with methanol due to residual methanol or methoxy groups in the pectic acid from which the ethyl ester was prepared.

Specificity of Purified Orange Pectinesterase

The results with purified orange PE (Table III) show that this enzyme has a very high degree of specificity for esters of polygalacturonic acid and confirm the finding (14) that acetylsterase is distinct from PE. Not only the methyl ester but also the ethyl ester of polygalacturonic acid is hydrolyzed by PE.

Fungal Pectinesterase

An interesting point of difference between the higher-plant and the fungal PE preparations is their actions on the methyl esters of galac-

TABLE III
*Specificity of Crude and Purified Orange Pectinesterase (PE) on
Several Non-Pectin Esters*

| | Esterase units Crude PE | $\times 10^3$ /pectinesterase unit Purified PE |
|---------------------------------|----------------------------|---|
| Methyl acetate | 150 | <2 |
| Methyl adipate | 150 | <2 |
| Methyl benzoate | 60 | <2 |
| Methyl <i>n</i> -butyrate | 360 | <2 |
| Methyl β -bromopropionate | 640 | 20 |
| Methyl formate | 500 ^a | 20 ^a |
| Dimethyl malonate | 390 | 10 |
| Methyl phenyl acetate | 220 | <2 |
| Methyl propionate | 100 | <2 |
| Methyl sebacate | 100 | <2 |
| Ethyl acetate | 200 | <2 |
| Ethyl formate | 280 | <2 |
| Ethyl <i>n</i> -butyrate | 310 | <2 |
| Ethyl lactate | 40 | 4 |
| Acetylcholine | 60 | <2 |
| Allyl acetate | 700 | <2 |
| Phenyl acetate | 2,000 | 80 |
| Phenyl propionate | 2,100 | 90 |
| Benzyl acetate | 870 | <2 |
| Monoacetin | 250 | <2 |
| Diacetin | 880 | 4 |
| Triacetin | 1,210 | 4 |
| Monoacetyl ethylene glycol | 360 | <2 |
| Diacetyl ethylene glycol | 690 | 8 |
| Monopropionin | 170 | <2 |
| Tripropionin | 440 | 7 |
| Methyl polygalacturonate | 47,000 | 50,000 |
| Ethyl polygalacturonate | 4,000 | 4,100 |

^a Results questionable because of extremely high chemical hydrolysis correction.

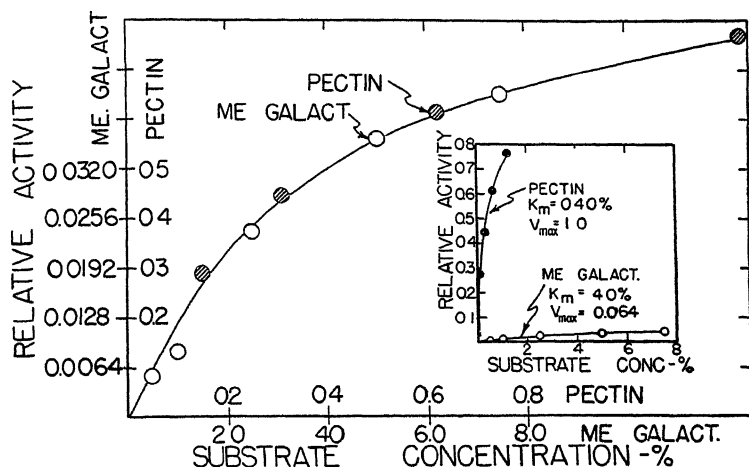


Fig. 3. The activity-substrate concentration function for the fungal enzyme preparation acting on pectin and on α -methyl-D-galacturonic acid methyl ester at pH 4.5.

turonic acid and on α -methyl-D-galacturonic acid; the higher-plant enzyme preparations do not hydrolyze these compounds while the fungal enzyme preparation does hydrolyze them at a significant though low rate, 0.2% and 0.54%, respectively, of the rate that it hydrolyzes pectin (0.5% substrate in each case). This, of course, may not be due to the PE in the fungal enzyme preparation.

TABLE IV

Hydrolysis of Nonpectin Esters at Two pH Values by Fungal Enzyme Preparation

| Substrate | Esterase units $\times 10^6$ /ml. of fungal PE preparation | |
|---|--|--------|
| | pH 4.5 | pH 6.8 |
| Methyl acetate | 3 | 40 |
| Methyl <i>n</i> -butyrate | 0.8 | 5 |
| Methyl malonate | 7 | 10 |
| Allyl acetate | 10 | 80 |
| Benzyl acetate | 9 | 120 |
| Phenyl acetate | 380 | 710 |
| Phenyl propionate | 160 | 320 |
| Triacetin | 30 | 310 |
| Tripropionin | 20 | 110 |
| Tributylin | 10 | 20 |
| α -Methyl-D-galacturonic acid methyl ester | 10 | 7 |

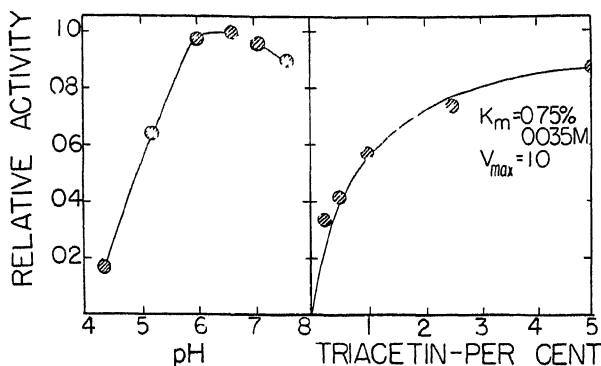


FIG. 4. The pH optimum at 0.5% substrate and the substrate concentration function for the fungal enzyme preparation acting on triacetin. The relative activity value of 1 on the pH curve is equivalent to value 0.4 on the substrate concentration curve. For the right-hand figure: Curve is theoretical and points are experimental.

Figure 3 shows the activity-substrate concentration relation for the fungal enzyme acting at pH 4.5 on pectin and α -methyl-D-galacturonic acid methyl ester. The K_m value of 4.0% for the simple substrate is 10 times the K_m value for pectin. The maximum velocity for the simple substrate is 7.1% of the maximum rate for pectin.

With respect to the effect of pH on activity, the hydrolysis of the galacturonic acid ester by the fungal preparation more nearly resembles pectin hydrolysis than nonpectin ester hydrolysis. Although pectin is much more rapidly hydrolyzed at pH 4.5 than at 6.8 (20) and the galacturonic acid ester is only slightly more rapidly hydrolyzed at pH 4.5 than at 6.8, yet the other esters are, in all cases studied less rapidly hydrolyzed at the lower than at the higher pH.

Nonpectin Esterase of the Fungal Preparation

The nonpectin esterase activity of the crude fungal enzyme apparently is due *partially* to an acylesterase (14). The two characteristics of the esterase studied, the pH optimum and activity-substrate concentration relationship (Fig. 4), are similar to those previously reported (14) for citrus acylesterase acting on triacetin. On the other hand, the relative rates of hydrolysis of methyl acetate and methyl butyrate by the orange enzyme is about 2.4, whereas for the fungal enzyme it is about 0.2.

DISCUSSION

From the data presented here it is likely that purified PE preparations from both higher-plant and fungal sources would hydrolyze pectin more than 5000 times as fast as they would hydrolyze any of the substrates studied (at 0.5% concentration) except those closely related to pectin. Obviously, this would correspond to at least a 500-fold greater rate even at 5% substrate concentration. It therefore appears that the polygalacturonide structure is essential for PE activity. Even the methyl ester of polymannuronic acid (alginic acid) was not hydrolyzed by PE. For the alcohol part of the ester, PE is not so specific. PE from the several sources hydrolyzes the ethyl ester of polygalacturonic acid 6–16% as fast as it does the corresponding methyl ester. This corresponds to 2.8–13.1% of the rate of hydrolysis of pectin (Table II). The esterase that acts on pectin is therefore not restricted to methyl esters. Only in the case of fungal PE is it possible that methyl galacturonate can be hydrolyzed by PE. Mills (21) found that a PE preparation from the bacterium *Pseudomonas prunicola* was able to hydrolyze tributyrin and other glycerides but not ethyl acetate. The data presented do not prove whether the activities on pectin and the glycerides were due to the same enzyme.

The nonpectin esterase activities are of interest principally because they indicate some of the esterase activities to be found in plants. Undoubtedly, acetylerase is responsible for much of the nonpectin esterase activity observed in the various plants used in this work. Even the fungal preparation appears to contain acetylerase, since the K_m and pH optimum for the hydrolysis of triacetin is about the same as for citrus acetylerase. It is possible that some of the very low activities observed may have been due to the hydrolysis of impurities in the substrates used. To indicate that this is not likely in most cases, the percentages of the substrate hydrolyzed in 24 hr. are given (upright numbers) in Fig. 1 above the citrus bars. Since the rate was practically linear during this time (unless more than 8% hydrolysis occurred in 6 hr.), the activities generally must have been due to action on the named compound instead of an impurity, unless a gross contaminant occurred.

In considering esterases other than PE it should be remembered that the enzyme preparations were made primarily to preserve PE activity. For example, with tomatoes, PE was prepared from solids from which "serum" had been separated. The "serum" contained only traces of

PE but did contain most of the acetylsterase. Nevertheless, the results are of interest because they show the broad nature of the specificity of plant esterases for simple esters and because they indicate certain differences in specificity.

The difference in the relative rates at which the four enzyme preparations attack methyl butyrate (also ethyl butyrate) and tributyrin is outstanding (Fig. 2). Also, the rates of action on phenyl acetate and phenyl propionate are strikingly higher than those on the other esters, as previously noted (14,22). If the hydrolysis of the two phenyl esters and methyl acetate (Fig. 2) is due to one enzyme in all four plants, it is obvious that they must differ markedly in detail character (K_m , V_{max} , or pH optimum) from plant to plant.

SUMMARY

Orange pectinesterase (PE) is highly specific. It hydrolyzes pectin at least 1000 times as fast as it does any of some 50 nongalacturonide ester tested. Methyl- and ethylpolygalacturonate, which have a lower molecular weight than pectin, were hydrolyzed at about 50% and 4%, respectively, of the rate of pectin hydrolysis. All nongalacturonate substrates hydrolyzed by crude orange PE were hydrolyzed not at all or at a greatly (20-fold) reduced rate by purified PE. Evidently the nongalacturonate esterase activities associated with orange PE preparations are due to other enzymes. Relative rates are for 0.5% substrate concentration in all cases.

Crude preparations of alfalfa, tomato, and fungal PE hydrolyzed pectin at least 60 times as fast as they did any substrates except phenyl acetate and phenyl propionate. The pectinesterase enzymes from these sources were not purified. The phenyl acetate, phenyl propionate, and other esterase activities of the orange PE preparation were shown to be different from PE. This is probably also true for the alfalfa, tomato, and fungal sources of PE.

The fungal PE preparation hydrolyzed both methyl-D-galacturonate and methyl- α -methyl-D-galacturonate at 0.2% and 0.54% of the rate it hydrolyzed pectin. Although this may be due to the PE in the preparation, PE from the higher-plant sources did not hydrolyze these esters at a significant rate.

The esterases (acetylsterase and probably other esterases) of higher plants and fungi can catalyze the hydrolysis of a large number of simple

esters but the specificity pattern (or spectrum) differs quantitatively, of course, from plant to plant.

Orange PE was purified over 100-fold (nitrogen basis) by salt fractionation and adsorption procedures.

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Inactivation Studies on Pancreatic Lipase. II. The Relation Between Substrate Structure and Inactivation Effect

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INTRODUCTION

Studies on the differential inactivation of hog pancreas homogenates and of fractions thereof by pretreatment of the enzymes with heat, alkali, and by digestion of a pancreas fraction with crystalline trypsin have shown the presence of an enzymatic component which is specific for the monovalent alcohol esters and another which is specific for the triesters of glycerol (1,2). While the former undergoes a decrease to a very low level of activity by any of the aforementioned inactivation procedures, the activity loss of the second is of a much smaller order of magnitude. The aim of the present study is to establish the relation between the inactivation effect and the substrate structure by employing substrates which represent a series of intermediate compounds between monovalent alcohol and trivalent alcohol esters with monocarboxylic acids. In addition, the enzymatic susceptibility of monovalent alcohol esters of some polycarboxylic acids has been studied.

As in the previous investigations the procedures have included the following: (a) differential heat inactivation of the glycerolated homogenates, (b) differential alkali inactivation of the aqueous homogenates, and (c) determination of the activity of fraction P_L of pancreas obtained by digestion and differential inactivation with crystalline trypsin. All the inactivation experiments were performed as described previously (1).

EXPERIMENTAL

The Substrates

The following three-carbon-chain alcohol esters have been used: tributyrin, α , β -dibutyryl, monobutyryl, α , β -propylene glycol dibutyrate, α -propylene glycol monobuty-

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rate, *n*-propyl-*n*-propionin, α,β -propyleneglycol dipropionate, *n*-propyl *n*-propionate, and in addition tributyl citrate, tributyl aconitate, dibutyl malonate, and dibutyl succinate. In Table I analyses and boiling points of those esters have been given which are not reported in the literature. The esters were prepared by heating corresponding amounts of the acid and alcohol to the boiling point of their mixture for 16 hr. in the presence of an excess of anhydrous CuSO_4 and 1 ml. of $\text{H}_2\text{SO}_4/150$ ml. of reaction mixture, followed by the usual purification procedure, *i.e.*, washing with bicarbonate solution, drying over Na_2SO_4 , and distillation *in vacuo* (3). While this method gave satisfactory results in most instances, the esters of propylene glycol were prepared by the interaction of the acyl chlorides with the alcohols in the presence of an excess of pyridine (2). Monopropionin was prepared by interaction of sodium propionate with glycerol- α -chlorohydrin at 180°C .²

TABLE I
Analyses and Boiling Points of Esters

| Substrate | Calcd. | | Found | | Collected at | |
|-------------------------------|--------|------|-------|------|--------------------|----------|
| | C | H | C | H | Temperature | Pressure |
| | | | | | $^\circ\text{C}$. | mm. Hg |
| Propylene glycol dibutyrate | 61.02 | 9.32 | 61.33 | 9.40 | 89-91 | 3 |
| Propylene glycol monobutyrate | 57.50 | 9.65 | 57.54 | 9.55 | 63-65 | 3 |
| Propylene glycol dipropionate | 57.37 | 8.56 | 57.50 | 9.00 | 65-67 | 3 |
| Monopropionin | 48.65 | 8.09 | 48.53 | 8.27 | 109-11 | 3 |
| Tributyl citrate | 59.97 | 8.92 | 59.39 | 8.79 | 178-80 | 3 |
| Tributyl aconitate | 63.07 | 8.82 | 63.27 | 8.96 | 167-68 | 3 |
| Dibutyl succinate | 62.55 | 9.62 | 62.56 | 9.69 | 116-17 | 3.5 |

The boiling points of the other esters were compared with those reported in the literature and their purity was ascertained by elemental analysis. For use in the enzyme tests all were emulsified in phosphate buffer of pH 8 with half their weight of gum arabic (with the exception of monobutyrin and monopropionin) and their concentration was adjusted so as to contain, respectively, 1 mmole of the acyl or the alcohol radical in 10 ml. of the emulsions of the polyvalent alcohol or polycarboxylic acid esters. To each 10 ml. of emulsion was added 3 ml. of the enzyme preparation.

Method of Assay

The titration method described before (1) was employed. Since many of the substrates possess a very high initial rate of hydrolysis the zero points were estimated by introducing first the ester emulsion and then the enzyme into neutralized formol in the same proportion as they are present in the final enzyme-substrate mixture. This precaution was necessary since it was observed that in the short time interval that

² α,β -Dibutyrin was prepared from redistilled α,β -dichlorohydrin (Eastman Kodak Co.) and sodium butyrate in a similar manner as monopropionin.

elapsed between the mixing of the substrate emulsion with the enzyme and the removal of an aliquot (about 15 sec.) some hydrolysis had already occurred, thus rendering the zero points too high.

The Enzymes

Glycerolated and aqueous hog pancreas homogenates and the fraction P_L obtained from pancreas by differential inactivation with crystalline trypsin (1) were employed as before.³

The results of the pretreatment with heat, crystalline trypsin, and alkali are given, respectively, in Tables II, III, and IV.⁴

DISCUSSION

With pancreatic preparations two types of ester hydrolysis under comparable experimental conditions may be observed and, consequently, must be attributed to two different enzymes. The first one, which hydrolyzes the triglycerides, acts upon these substrates with a high initial rate of hydrolysis, which, however, slows down very soon and never, under the present conditions, brings about more than a 40% hydrolysis of the esters. This enzyme possesses a relatively high resistance toward inactivation by either one of the procedures mentioned above. The inactivation seems rather to be concerned with a decrease in the capacity to hydrolyze the substrates at their former high *initial* velocity, than to fail to bring about their hydrolysis at all. The other, more labile enzyme, hydrolyzes the monohydric alcohol esters with a lower initial rate of velocity which is maintained until the hydrolysis has reached 30%; thereafter, the time curves of the hydrolysis cease to remain linear, but continue at least to 90% cleavage. Treatment of this enzyme with heat, alkali, or crystalline trypsin results in an almost complete destruction of the hydrolytic capacity.

³ The enzymatic behavior of the alkali-treated crude homogenate is similar to that of the purified fraction P_I (1) except at the highest alkalinity used (pH 10-11) where the activity loss of P_I exceeded that of the crude homogenate. Since fraction P_I suffers a decrease in activity after 3 days storage at 4°C. the more stable crude homogenate has been used instead.

⁴ It has been found in later experiments that P_L preparations obtained by increasing the trypsin from 156 mg./1300 mg. of enzyme nitrogen (260 ml. of P_I suspension) to 234 mg. fail completely to hydrolyze monovalent alcohol esters. In this case, however, the general decrease in enzymatic activity renders the estimation of smaller activity differences in the above substrate series too difficult. Moreover, incubation of fraction P_I with trypsin should not be extended over the 3-hr. limit owing to its not inconsiderable thermal lability which causes about 30% of activity loss in the trypsin-free control if incubation is extended beyond this time.

All the polyhydric alcohol esters used in this study are attacked by the enzymes with a rather high initial rate of hydrolysis which, however, slows down rapidly after having brought about a 5-8 per cent cleavage of the esters. In this respect the course of their hydrolysis is very similar to that of the triglycerides. This is best exemplified in Fig. 1, presenting the enzymatic behavior of the heat treated glycerolated homogenates towards α -propylene glycol monobutyrate on the one hand and towards *n*-propyl *n*-butyrate on the other. Although the decrease in hydrolysis by the heated homogenate as calculated from the linear part of

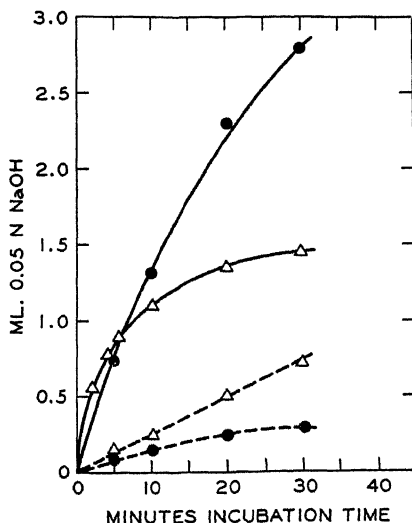


Fig. 1. Hydrolysis of α -propylene glycol monobutyrate and of *n*-propyl *n*-butyrate by heat treated and by the nontreated glycerolated homogenates. Concentration of the enzymes: 0.777 mg. N/ml. Broken lines indicate hydrolysis by homogenate after heat treatment. Δ α -Propylene glycol monobutyrate, \bullet *n*-propyl *n*-butyrate.

the curves is of the same order of magnitude for both esters (Table II) the extent of the cleavage of the propylene glycol ester is considerably higher than that of the propyl alcohol ester even in a rather short time interval. The rather prolonged linear part of the curves produced by the hydrolysis of the propylene glycol ester by the heat-treated homogenate is shared by all the polyhydric alcohol esters used, and is not characteristic of the heat-treated enzymes only but also of the alkali- and trypsin-treated preparations as well.

Table II shows a steady decrease in the residual activity of the heat-treated homogenate toward the esters of the polyhydric alcohol series employed. This decrease corresponds to the decrease of ester linkages in the glycerol and propylene glycol ester molecules and, moreover, shows that of all the homologous esters employed, the triglycerides suffer the smallest percentage losses in the hydrolysis by the heat-

TABLE II

Hydrolysis of the Esters by Heated and Nonheated Glycerolated Homogenates

Activities are calculated from the linear part of the time curves and are expressed in μ moles of NaOH consumed/hr./mg. of enzyme nitrogen. Glycerol extract was diluted with phosphate buffer so as to contain 1.554 mg. N/ml. for monopropionin and *n*-propyl *n*-propionate, and 0.777 mg. N/ml. for all the other esters.

| Substrate | Nonheated homogenate | Heated homogenate | Decrease in hydrolysis |
|---|-------------------------|----------------------|---------------------------|
| | <i>activity</i> | <i>activity</i> | <i>per cent</i> |
| Tributyryn | 1333 | 512 | 62 |
| α,β -Dibutyryn | 1601 | 356 | 78 |
| α,β -Propylene glycol dibutyrate | 2257 | 356 | 84 |
| Monobutyryn | 945 | 135 | 86 |
| α -Propylene glycol monobutyrate | 1395 | 210 | 85 |
| <i>n</i> -Propyl <i>n</i> -butyrate | 670 | 42 | 96 |
| Tripropionin ^a | 2657 | 741 | 72 |
| α,β -Propylene glycol dipropionate | 1445 | 236 | 84 |
| Monopropionin | 194 | 0 | 100 |
| <i>n</i> -Propyl <i>n</i> -propionate | 150 | 0 | 100 |
| Dibutyl malonate | 337 | 0 | 100 |
| Dibutyl succinate | 377 | 0 | 100 |

^a The activities of both the heat-treated and the nontreated homogenates towards tripropionin and monobutyryn are considerably higher than those given earlier (1). As was noted only recently, the rate of hydrolysis of these esters is extremely high in the first 30 sec. of their enzymatic hydrolysis. The zero values were therefore estimated as described under *Method of Assay*, and the author wishes to correct herewith the values given earlier.

treated homogenate. The results obtained by alkalization and trypsin treatment (Fraction *P_L*) of the homogenates are essentially similar (Tables III and IV).

Comparison of the enzymatic susceptibility of the butyl alcohol esters of malonic and of succinic acid with that of the butyric acid esters of dihydric alcohols shows that the activity of the heat-, alkali-, or trypsin-

TABLE III

Hydrolysis of the Esters by Aqueous Homogenates and by Fraction P_L

Activities are expressed as in Table II. Concentration of P_L: For *n*-propyl *n*-butyrate, propylene glycol monobutyrate, α,β -dibutylin, and dibutyl succinate 0.915 mg. N/ml.; for α,β -propylene glycol dibutyrate 0.591 mg. N/ml.; for dibutyl malonate and tributyrin 0.660 mg. N/ml.; for monobutylin 1.000 mg. N/ml.; and for monopropionin and propyl *n*-propionate 2.000 mg. N/ml. Concentration of homogenate: 0.460 mg. N/ml.

| Substrate | Pancreas homogenate | Fraction P _L | Decrease in hydrolysis |
|---|---------------------|-------------------------|------------------------|
| | <i>activity</i> | <i>activity</i> | <i>per cent</i> |
| Tributylin | 2723 | 980 | 64 |
| α,β -Dibutylin | 2986 | 658 | 78 |
| α,β -Propylene glycol dibutyrate | 4019 | 766 | 81 |
| α -Propylene glycol monobutyrate | 2151 | 311 | 85 |
| Monobutylin | 1741 | 243 | 85 |
| <i>n</i> -Propyl <i>n</i> -butyrate | 1464 | 50 | 97 |
| Tripropionin | 5044 | 2000 | 60 |
| α,β -Propylene glycol dipropionate | 3094 | 533 | 83 |
| Monopropionin | 386 | 40 | 90 |
| <i>n</i> -Propyl <i>n</i> -propionate | 319 | 29 | 91 |
| Dibutyl malonate | 840 | 0 | 100 |
| Dibutyl succinate | 840 | 0 | 100 |

TABLE IV

Hydrolysis of the Esters by Homogenates Treated to pH 10.5 and by the Nontreated Control

Activities are calculated as in Table II. Enzyme concentration: For dibutyl malonate and dibutyl succinate 1.020 mg. N/ml.; for monopropionin and *n*-propyl *n*-propionate 2.012 mg. N/ml.; for all other esters 0.500 mg. N/ml.

| Substrate | Nontreated homogenate | Treated to pH 10.5 | Decrease in hydrolysis |
|---|-----------------------|--------------------|------------------------|
| | <i>activity</i> | <i>activity</i> | <i>per cent</i> |
| Tributylin | 2723 | 1842 | 32 |
| α,β -Dibutylin | 2968 | 1456 | 51 |
| α,β -Propylene glycol dibutyrate | 4019 | 2630 | 35 |
| Monobutylin | 1741 | 617 | 65 |
| α -Propylene glycol monobutyrate | 2151 | 1430 | 40 |
| <i>n</i> -Propyl <i>n</i> -butyrate | 1464 | 237 | 84 |
| Tripropionin | 5044 | 4641 | 7 |
| α,β -Propylene glycol dipropionate | 3094 | 2506 | 19 |
| Monopropionin | 386 | 141 | 64 |
| <i>n</i> -Propyl <i>n</i> -propionate | 319 | 108 | 66 |
| Dibutyl malonate | 685 | 0 | 100 |
| Dibutyl succinate | 795 | 78 | 91 |

treated homogenates has been reduced toward the former either to zero or to a very low level. Moreover, tributyl citrate and tributyl aconitate fail to be hydrolyzed at all by hog pancreas homogenates.

The residual activity in the heat-, alkali-, and trypsin-treated homogenates is thus seemingly concerned with the polyhydric alcohol and not with the polycarboxylic acid esters.

ACKNOWLEDGMENT

The author is indebted to Mr. R. J. Koegel for the elemental analyses.

SUMMARY

The relation between the substrate structure and the rate of hydrolysis of various polyhydric and monovalent alcohol esters with butyric and propionic acid by differentially inactivated hog pancreas homogenates has been investigated.

Percentage losses in the initial rates of the hydrolysis of the 3-carbon-chain alcohol esters increased in the following order: tributyrin, α,β -propylene glycol dibutyrate, *n*-propyl *n*-butyrate; and in the butyrin series: tributyrin, dibutyrin, and monobutyrin. Similarly, the loss in hydrolysis suffered by α -propylene glycol monobutyrate exceeded that of α,β -propylene glycol dibutyrate. Analogous results have been obtained with the propionic acid ester homologs.

Butyl alcohol diesters of malonic and of succinic acid fail to be hydrolyzed by pancreas homogenates heated for 25 min. to 60°C. and by pancreas fractions treated with crystalline trypsin. Their enzymatic susceptibility is thus markedly different from that of the diesters of dihydric alcohols. Moreover, tributyl esters of citric and of aconitic acid are not hydrolyzed by hog pancreas homogenates. The comparatively high residual activity of the differentially inactivated homogenates is thus specifically concerned with the polyhydric alcohol esters.

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Studies on the Biological Action of Antimycin A¹

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INTRODUCTION

The isolation, crystallization, and certain properties of a new antibiotic, antimycin A, from the fermentation broth of an undetermined species of *Streptomyces* was reported earlier from this laboratory (1). It was found to be a nitrogenous phenol of the probable empirical formula, $C_{28}H_{40}O_9N_2$.

In the present paper, the effect of antimycin A on the growth and metabolism of yeast and its action on certain enzymes are described. Enzyme inhibition studies were centered on the succinoxidase system. Preliminary toxicity studies with rats are also reported.

EXPERIMENTAL

Effect of Antimycin A on Yeast Growth

Ten ml. of the synthetic medium of Olson and Johnson (2) previously sterilized at 120°C. for 10 min. was inoculated with *Saccharomyces cerevisiae* Y 30 and incubated for 24 hr. at 30° with continuous shaking.³ One ml. of this yeast preparation was diluted to approximately 25 ml. with sterile redistilled water, the exact dilution being adjusted to give a reading of 80–85 on the Evelyn photometer with 660 m μ filter. One drop of this suspension was used to inoculate the experimental flasks.

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³ A culture of *Saccharomyces cerevisiae* Y 30 was kindly furnished by Dr. M. J. Johnson.

Twenty-five-ml. portions of the synthetic medium were placed in 500-ml. Erlenmeyer flasks and sterilized for 10 min. at 120°C. On cooling, the desired amount of antimycin A was added aseptically from a stock solution containing 1 $\mu\text{g.}/\text{ml.}$ in 95% ethanol. The flasks were then inoculated with the yeast suspension prepared above. After 24 hr. incubation at 30° with continuous shaking, the amount of growth was estimated by turbidity determinations on the Evelyn photometer at 660 $\text{m}\mu$. The results of a representative experiment are shown in Fig. 1.

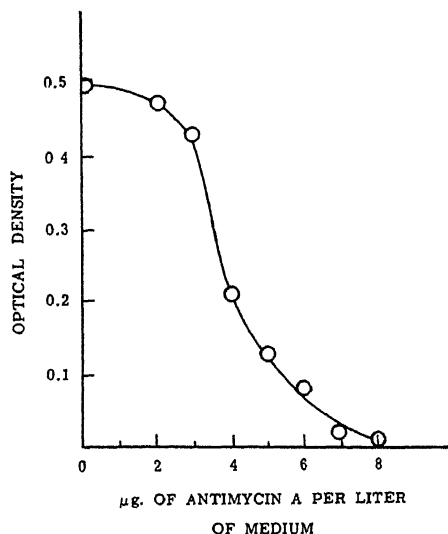


FIG. 1. Effect of antimycin A on growth of *Saccharomyces cerevisiae* Y 30 on a synthetic medium.

Six ml. of a natural medium (40 g. glucose, 10 g. peptone, 3 g. yeast extract in 1 l. of water, pH 7.0) was sterilized for 10 min. at 120°C. After cooling, this was inoculated with *S. cerevisiae* Y 30 and incubated for 12 hr. at 25°C. without shaking. One ml. of the culture was diluted to 25 ml. with sterile redistilled water. The suspension of yeast cells was adjusted to give a reading of 80–85 on the Evelyn photometer at 660 $\text{m}\mu$. One drop of this suspension was used to inoculate the experimental flasks below.

Ten ml. of the natural medium was placed in 6-oz. rectangular culture bottles and sterilized at 120°C. for 10 min. On cooling, the desired

amount of antimycin A was added aseptically from a stock solution containing 1 $\mu\text{g.}/\text{ml.}$ in 95% ethanol. The flasks were then inoculated with the yeast suspension prepared above. After 24 hr. standing at 25°C., turbidity measurements showed no inhibition of yeast growth even at concentrations of 40 $\mu\text{g.}/\text{l.}$ of antimycin A.

Effect of Antimycin A on Yeast Metabolism

S. cerevisiae Y 30 cells grown on the synthetic medium as described above were centrifuged, washed once with distilled water, and then resuspended in a sufficient volume of fresh culture medium to obtain a reading of 55–60 on the Evelyn photometer at 660 $\text{m}\mu$. The Warburg respirometer was used and the experiments were carried out in air at

TABLE I

Composition of Fermentation Medium

The following ingredients were dissolved in 300–400 ml. of water, the solution adjusted to pH 4.6 and diluted to 500 ml. with distilled water.

| Component | Amount g. |
|---|--------------|
| Acetic acid | 1.50 |
| Sodium acetate | 2.05 |
| KH_2PO_4 | 1.36 |
| Glucose | 7.92 |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.324 |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ | 0.102 |
| Thiamine hydrochloride | 0.1686 |
| Niacin | 0.100 |
| Pyridoxine | 0.05 |
| Calcium pantothenate | 0.05 |

30°C. Parallel experiments for the measurement of total carbon dioxide output and oxygen uptake were done using the same levels of antimycin A. To each flask were added 1.5 ml. of fermentation medium⁴ (Table I), the desired amount of antimycin A in aqueous suspension (1 $\mu\text{g.}/\text{ml.}$), water to make 2.0 ml., and lastly 1 ml. of the yeast suspension prepared as stated above. Two-tenths ml. of 20% potassium hydroxide was placed in the center well of the flasks used for the measurement of oxygen uptake together with a 1 sq. in. piece of fluted ashless filter paper for carbon dioxide absorption. Typical results are shown in Fig. 2.

⁴ The composition of the fermentation medium was suggested by Dr. H. A. Lardy

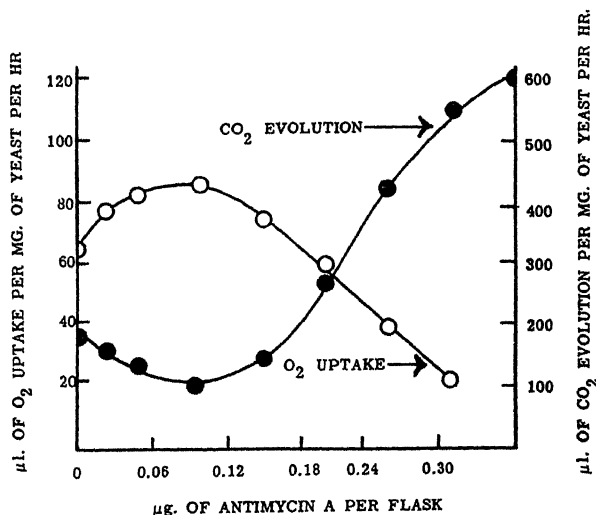


Fig. 2. Effect of antimycin A on aerobic metabolism of *S. cerevisiae* Y 30.

To determine the effect of antimycin A on anaerobic metabolism of yeast, Warburg flasks having a side arm equipped with a gas vent were used. To each were added 1.5 ml. of the fermentation medium, 0.5 μ g. of antimycin A in aqueous suspension, water to make 2.0 ml., and lastly 1.0 ml. of yeast suspension. A slow stream of nitrogen was passed through the flasks for 12 min. to flush out all oxygen. A control was run under aerobic conditions with antimycin A and another anaerobically but without antimycin A. In Table II results of this experiment are given. It may be seen that there was no effect of antimycin A on the fermentation of yeast carried out in an atmosphere of nitrogen and that the aerobic fermentation in the presence of antimycin A (0.5 μ g./flask) matched the anaerobic level.

TABLE II

Effect of Antimycin A on Anaerobic Metabolism of S. cerevisiae Y 30

| Atmosphere in the experimental flask | Amount of antimycin A/flask (3 ml.) μ g. | Carbon dioxide output/mg. yeast/hr. ^a μ l. |
|--------------------------------------|---|--|
| Air | 0.5 | 700 |
| Nitrogen | none | 710 |
| Nitrogen | 0.5 | 698 |

^a Based on 45 min. observation following 10 min. equilibration.

Effect of Antimycin A on the Succinoxidase System

The measurements were made in the Warburg respirometer at 37° and in an air atmosphere by the procedure of Schneider and Potter (3). The source of the succinic dehydrogenase was a 10% rat liver homogenate in redistilled water prepared by means of the Potter-Elvehjem homogenizer (4). To each flask were added 1 ml. of 0.1 *M* phosphate buffer (pH 7.4), 0.4 ml. of 0.0001 *M* cytochrome c, 0.3 ml. of 0.004 *M* calcium chloride, 0.3 ml. of 0.004 *M* aluminum chloride, 0.3 ml. of 0.5 *M* sodium succinate (pH 7.4), the desired amount of antimycin A (1 μ g./ml. in 50% ethanol), and redistilled water to make 2.8 ml. Controls containing the above components with the substitution of 0.02–0.06 ml. of 50% ethanol in place of the antimycin A solutions were also included to test for any alcohol inhibition.

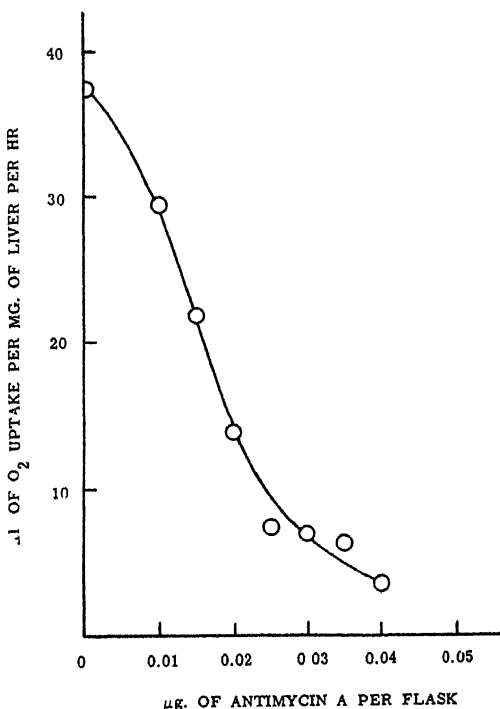


FIG. 3. Inhibition of liver succinoxidase by antimycin A. The final volume in each flask was 3 ml.

Two-tenths ml. of a 10% liver homogenate was then added to each flask. Lastly, 0.2 ml. of 20% aqueous potassium hydroxide was added to the center well of each flask together with a fluted piece of ashless filter paper (1 sq. in.) for carbon dioxide absorption. Ten min. was allowed for equilibration; oxygen uptake readings were then taken over periods of 30–60 min. Values for oxygen uptake/mg. of dry tissue/hr. were calculated from the "10/20 ratio," the average uptake/20 mg. of fresh tissue/10 min. No inhibition due to the ethanol was observed. In Fig. 3, the Q_{O_2} values are plotted against varying levels of antimycin A.

TABLE III
*Effect of Substrate Concentration on the Inhibition
of Succinoxidase by Antimycin A*

| Antimycin A/flask ^a | Volume of 0.5 M succinate/flask ^a | Oxygen uptake/mg. of tissue/hr. ^b |
|-----------------------------------|---|---|
| $\mu\text{g.}$ | ml. | $\mu\text{l.}$ |
| 0.03 | 0.15 | 13 |
| | 0.30 | 13 |
| | 0.45 | 15 |
| 0.04 | 0.15 | 9 |
| | 0.30 | 8 |
| | 0.45 | 7 |

^a Final volume, 3 ml./flask.

^b These values are based on 30 min. observation following 10 min. equilibration at 37°.

The effect of substrate concentration on the inhibition of succinoxidase by antimycin A was tested by varying the concentration of succinate while the concentration of the inhibitor (antimycin A) and the enzyme were kept constant. The results of these experiments are given in Table III. Experiments were also carried out in which varying amounts of enzyme were used with a given level of substrate and inhibitor. Typical results are presented in Fig. 4. The amount of succinate used was again 0.3 ml. of 0.5 M solution, and the final volume in each Warburg vessel was 3.0 ml.

*Succinic Dehydrogenase and Antimycin A in
Thunberg Experiments (5)*

In each Thunberg tube were placed 2.0 ml. of 0.067 M phosphate buffer (pH 7.0), 2.0 ml. of 0.05 M sodium succinate (pH 7.0), 1 ml. of

0.0001 *M* methylene blue, the desired amount of antimycin A (80 $\mu\text{g.}$ /ml. in 95% ethanol), and water to make 5.5 ml. One ml. of 1% rat liver homogenate was then placed in the side-arm cap. The total volume was 6.5 ml. The tube and side arm were then lightly greased with a high-vacuum preparation and connected. The tubes were simultaneously evacuated by means of a good water pump for 3 min., good agitation

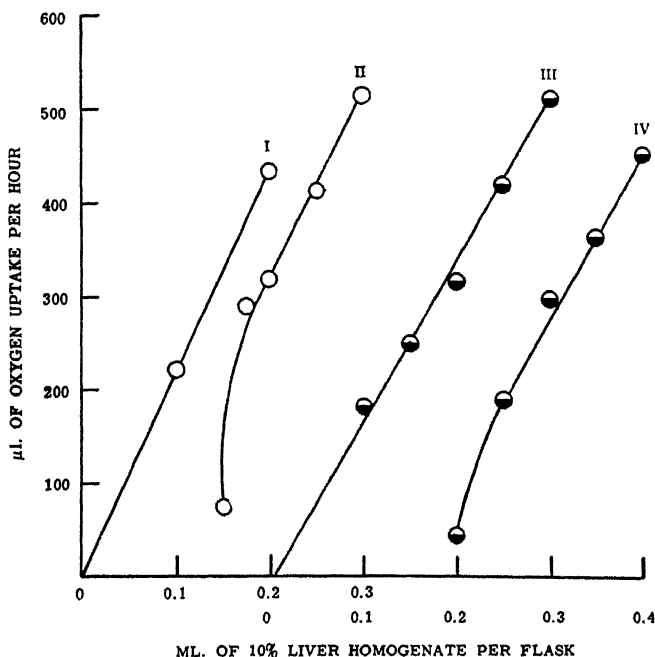


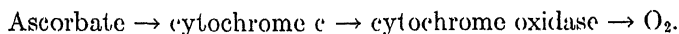
FIG. 4. Effect of antimycin A on succinoxidase activity at various inhibitor and enzyme levels. The substrate in all cases was 0.3 ml. of 0.5 *M* succinate/flask (final volume, 3 ml.). Curves I and III, controls. Curve II, 0.015 $\mu\text{g.}$ of antimycin A/flask. Curve IV, 0.030 $\mu\text{g.}$ of antimycin A/flask.

being provided throughout this period. After a 10-min. period of equilibration in a constant-temperature bath at 37°, the contents of tube and cap were thoroughly mixed and the "zero" time reading taken on the Evelyn photometer with 660 $m\mu$ filter. The tubes were quickly returned to the constant-temperature bath, and the reduction followed colorimetrically at 5-min. intervals for a period of 1 hr.

The results of these inhibition studies are given in Fig. 5.

Effect on Cytochrome Oxidase

The effect of antimycin A on the cytochrome system was investigated by means of the following reaction (6):



The measurements were made at 37° using the Warburg respirometer. To each flask were added 1 ml. of 0.1 *M* phosphate buffer (pH 7.4), 0.5 ml. of 0.0004 *M* cytochrome *c*, 0.3 ml. of 0.004 *M* calcium chloride, 0.3 ml. of 0.004 *M* aluminum chloride, 0.3 ml. of 0.114 *M* sodium ascorbate (pH 7.0), the desired amount of antimycin A (aqueous suspension), and water to make 2.85 ml. Fifteen-hundredths ml. of a 1% rat

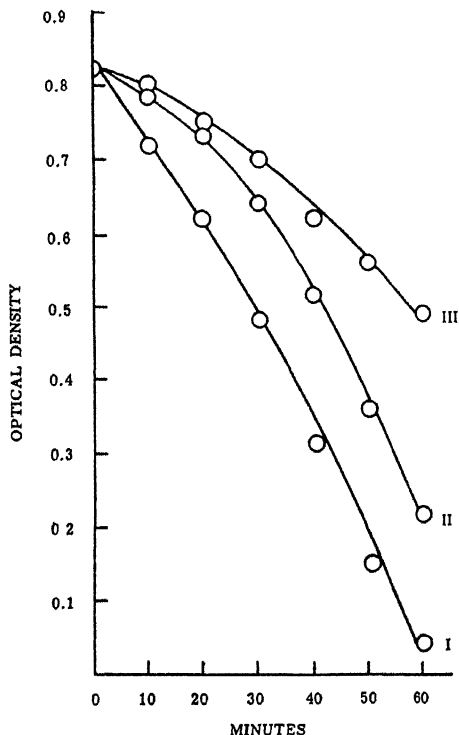


FIG. 5. Inhibition of succinic dehydrogenase activity by antimycin A in Thunberg experiments. Curve I, control. Curve II, 6 $\mu\text{g.}$ of antimycin A/tube (final volume 6.5 ml.). Curve III, 10 $\mu\text{g.}$ of antimycin A/tube.

TABLE IV
Effect of Antimycin A on Cytochrome Oxidase

| Antimycin A/flask ^a μg. | Oxygen uptake/mg. of liver/hr. ^b μl. |
|---------------------------------------|---|
| None | 280 |
| 0.5 | 283 |
| 1.0 | 278 |
| 10.0 | 280 |

^a Final volume, 3 ml./flask.

^b Calculated on 30 min. observation after 10 min. equilibration.

liver homogenate was then added. Lastly 0.2 ml. of 10% potassium hydroxide was added to the central well of each flask which also contained a 1-sq. in. piece of folded filter paper to facilitate absorption of carbon dioxide. The results are given in Table IV. It may be seen that antimycin A did not have any effect on this system, even when its concentration was as high as 3.3 μg./ml.

Reaction with Thiol Compounds

The procedure employed was similar to that described by Cavallito (7). Antimycin A was incubated with 2 *M* and 10 *M* proportions of cysteine, glutathione, thioglycolic acid, and 2,3-dimercapto-1-propanol (British anti-lewisite), respectively, at 25°C. and pH 7.0 in the presence of potassium phosphate buffer for 24 hr. Following the period of incubation, an agar-plate assay for the remaining antibiotic potency was

TABLE V
*Antibiotic Potency of Antimycin A After Incubation
with Various Mercapto Compounds*

| Thiol compound used ^a | Molar proportion of the thiol compound | Potency found per cent of original |
|----------------------------------|---|---------------------------------------|
| None | — | 100 |
| Cysteine | 2 | 120 |
| Cysteine | 10 | 105 |
| Glutathione | 2 | 95 |
| Glutathione | 10 | 130 |
| Sodium thioglycolate | 2 | 95 |
| Sodium thioglycolate | 10 | 85 |
| 2,3-Dimercapto-1-propanol | 2 | 140 |
| 2,3-Dimercapto-1-propanol | 10 | 105 |

^a The thiol compounds did not have any fungicidal activity when tested alone.

carried out. The results which are given in Table V indicate that these thiol compounds had no effect on the antibiotic potency of antimycin A.⁵

Toxicity of Antimycin A for Rats

Twelve rats weighing between 92 g. and 110 g. were used for this experiment. They were divided into two groups of six, each averaging 98 g. One group served as control. In the first week both groups were given stock ration and the average food intake was 15 g./day/rat. The average gain of body weight per rat was 33 g. and 34 g., respectively. During the second week one group was given antimycin A. Five mg. of antimycin A contained in 1 ml. of alcohol was thoroughly mixed with 5 g. of ration and fed to the rats. The rest of the ration was supplied without antimycin A later in the day after the rats had eaten all or most of the antimycin-treated ration. Records were kept of the food consumption and antimycin ingestion of these rats during the week. The control rats were given the same diet as in the first week. Results are given in Table VI.

TABLE VI
Oral Toxicity of Antimycin A to Rats

| | Group 1 ^a g. | Group 2 ^a g. |
|--|----------------------------|----------------------------|
| Average weight at start of experiment | 98 | 92 |
| Weight at end of first week (stock ration) | 131 | 126 |
| Rate of growth/week/rat | 33 | 34 |
| Total amount of antimycin A consumed/rat during second week ^b | 0.0238 ^c | None |
| Average food intake/rat/day | 7 | 15 |
| Weight (average) at end of second week | 96 | 158 |
| Changes in weight over second week | -35 | 32 |

^a Six rats in each group.

^b The range of variation of antimycin A intake by individual rats was 22.5-25 mg.

^c This intake corresponds to a total dosage of 181.6 mg./kg. based on the average starting body weight.

Two of the antimycin-treated rats were killed by decapitation and autopsied. All internal organs excepting the lungs appeared to be normal. In the lungs dark red patches were observed scattered throughout the organ. About 5% of the lungs were so afflicted. The remaining four rats of this group were then returned to the stock ration and were found to resume normal growth. During the following week, the average gain

⁵ The antibiotic assays were kindly carried out by Dr. Curt Leben.

of weight was 40 g./rat. They were kept under observation for 45 days after the termination of the experiment and were apparently normal in every respect.

Of the six rats (average 158 g.) used as controls up to this time, two were then given 20 mg., and two, 5 mg. of antimycin A dissolved in 1 ml. of methyl laurate. These solutions were administered by stomach tube. The remaining two rats were given only 1 ml. each of methyl laurate (no antimycin). Rats receiving 20 mg. died within 25 hr. showing symptoms of respiratory difficulties (panting) and those receiving 5 mg. died in 48 hr. The two controls given only methyl laurate remained unaffected. These were later given 2 mg. each of antimycin A by stomach tube as above, and survived without apparent ill effects.

DISCUSSION

The finding of Leben and Keitt (8) that antimycin A is toxic to many fungi suggested that it might affect the growth of yeast. On a synthetic medium, growth of *S. cerevisiae* Y 30 was completely inhibited by 8 μ g. of antimycin A/l. ($1.5 \times 10^{-8} M$), Fig. 1, but on a natural medium containing peptone and yeast extract no effect was observed at concentrations up to 40 μ g./l. The reason for this interesting difference has not yet been investigated.

The results plotted in Fig. 2 show that the antibiotic stimulated the aerobic fermentation of *S. cerevisiae* and inhibited its respiration. The two effects were so related that the former appeared to be a consequence of the latter. This conclusion is also supported by the data in Table II which show that the aerobic fermentation was not raised by antimycin A above the anaerobic level, and that the antibiotic had no effect on anaerobic glycolysis. At very low levels there was some stimulation of respiration and concomitant depression of fermentation (Fig. 2), as has been observed, for example, with phenylhydrazine (9).

The above findings suggested the possibility that antimycin A might block one or more enzyme systems of the tricarboxylic acid cycle. When its effect on the succinoxidase system was tested, extensive inhibition was observed (Fig. 3). The enzyme affected appeared to be succinic dehydrogenase, since cytochrome oxidase was not inhibited, whereas the Thunberg results plotted in Fig. 5 show almost complete inhibition at a concentration of $3 \times 10^{-6} M$ (10 μ g./tube, curve 3, Fig. 5).

It will be noted that higher concentrations of antimycin A were required to block succinic dehydrogenase activity, as measured by the

Thunberg technique, than were needed for inhibition of succinoxidase. The effective amounts per unit weight of liver tissue were, in fact, some 600–700 times greater in the former case. Whether this difference is attributable to the use of methylene blue in place of oxygen as the hydrogen acceptor, or to the inhibition of some other component of the succinoxidase system by antimycin A cannot be stated at present.

Several antibiotics have been suggested by Cavallito (7) and others as functioning by virtue of their ability to interact with mercapto compounds. Since the activity of succinic dehydrogenase appears to depend on the presence of intact —SH groupings (10), it seemed that antimycin A might also act through this mechanism. However, as indicated above, no effect was noted on anaerobic glycolysis in yeast, a process which involves the participation of a number of mercapto enzymes (11). Furthermore, incubation of the antibiotic with an excess of several —SH compounds did not reduce its antifungal potency (Table V) or its inhibitory power for succinoxidase. For these reasons it appears that the mechanism involved in the action of antimycin A probably is not concerned with —SH groups.

The inhibition of succinoxidase by antimycin A likewise does not fall in the category of metabolite–antimetabolite competition. This is shown by the failure of increasing amounts of substrate to reverse the inhibition (Table III), and particularly by the dependence of the extent of inhibition on the enzyme concentration (Fig. 4).⁶ It will be noted that plotting the rate of oxygen uptake against increasing amounts of homogenate in the absence of antimycin A gave straight lines passing through the origin. However, in the presence of the inhibitor, curves were obtained which were linear above a certain homogenate level, parallel to the control curve in this linear region, and displaced along the enzyme axis by an amount proportional to the amount of inhibitor present. Such behavior is characteristic of irreversible, noncompetitive inhibition (12).

The reason for the unexpectedly great inhibition at the lowest homogenate levels (Fig. 4, curves 2 and 4) is not clear. This effect was checked several times and was consistently observed. It may be the result of a partial metabolism of the antibiotic, occurring to a greater extent at the higher homogenate levels.

⁶ The authors wish to thank Dr. V. R. Potter for advice in connection with this experiment.

Previously investigated inhibitors of the succinoxidase system, excluding those affecting cytochrome oxidase, fall into two classes, viz., competitive inhibitors such as malonate and oxalacetate (13), and a miscellaneous list of noncompetitive inhibitors which probably owe their effectiveness to interaction with —SH groups (14). As indicated above, the action of antimycin A apparently differs from that of both types and the antibiotic is further distinguished by its much higher activity on a molar basis. While the most potent members of the former groups caused 50–80% inhibition at 10^{-5} *M* concentration, antimycin A under similar although not identical conditions caused over 80% inhibition at approximately 2×10^{-8} *M* (Fig. 3, 0.03 μ g./flask).

The preliminary studies carried out during the present investigation on the toxicity of antimycin A for rats have served to emphasize the importance of the mode of administration. Ingestion with the ration of 181.6 mg./kg. of body weight during one week (Table VI) resulted in no deaths, but the food intake and body weight of the animals was sharply reduced. However, no permanent damage appeared to result since the rats resumed normal growth and appeared healthy after being returned to the stock diet. In contrast a single dose of 125 mg./kg. administered by stomach tube as a solution in methyl laurate was almost immediately fatal, and even 30 mg./kg. caused death within 48 hr. in the two animals tested. Only the smallest dose given in this manner, 12 mg./kg., was tolerated without evidence of ill effects. In this connection it may be noted that the *LD* 50 value for antimycin A found by subcutaneous injection into mice was approximately 25 mg./kg. of body weight (15).

Studies of the effect of antimycin A on insects (16,17) have shown it to be highly toxic for some species and relatively innocuous for others. It is also known to be effective against a number of fungi, but without action on most of the bacteria tested (8). Thus a number of aerobic organisms are resistant to this antibiotic. Such cases are probably to be explained either on the supposition that the antimycin A does not actually penetrate the cell, or that it is previously inactivated by reaction with some other substance.

SUMMARY

Antimycin A, a new crystalline antibiotic from an unidentified species of *Streptomyces* has been found to inhibit strongly yeast growth in a synthetic, but not in a natural, medium. Aerobic respiration of the

yeast was stopped, and the fermentation stimulated to the anaerobic level. The substance was also found to inhibit the succinoxidase system where its effect apparently was exerted on succinic dehydrogenase. Antimycin A is the most potent inhibitor of succinoxidase so far discovered, and acts in a different manner from other known inhibitors of this enzyme system in that it is noncompetitive and not involved in reaction with —SH groups.

The toxicity of antimycin A to rats was found to vary with the mode of administration. When given by stomach tube in methyl laurate solution, 12 mg./kg. was tolerated, but 30 mg./kg. proved fatal.

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Effect of Xanthophylls on Utilization of Carotene by Chicks

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INTRODUCTION

Several papers have reported that xanthophylls lower the effectiveness of carotene or vitamin A for growth of rats and for storage of vitamin A in their livers (1,2,3). Chicks differ from rats in that they are able to store xanthophylls in certain tissues of their body. Xanthophylls therefore may not affect the utilization of carotene by the chick in the same degree or manner that they affect the utilization by the rat. Also speculation has been made regarding the effect of xanthophylls on relatively low levels of carotene (4).

The experiments reported in this paper were devised to determine the effect of xanthophylls on the utilization of carotene for storage of vitamin A in the livers of chicks, the effect of the level of xanthophylls on carotene utilization, and the effect of the level of carotene on the depressing action of xanthophylls.

EXPERIMENTAL

Oil solutions of carotene were prepared from commercial carotene purified by precipitation from benzene with methanol. Chromatographic analysis of the purified carotene showed it to contain 84.6% of β -carotene and 15.2% of α -carotene. In calculating the β -carotene equivalent of this mixture the β -carotene was considered 100% available and the α -carotene 25% available for liver storage of vitamin A (5). Xanthophylls were extracted from freshly prepared dehydrated alfalfa meal with methanol. The major portion of the carotene fraction was removed from the extract by phase separation between 90% methanol and Skellysolve B. The crude xanthophylls were removed from the methanol by extraction with ethyl ether. The ether solution was concentrated *in vacuo* and an equal volume of Skellysolve B was added. This mixture was chromatographed twice on Ca(OH)_2 . The carotenes passed through the column and the xanthophylls remained in the upper portion. The two major bands of xanthophylls were eluted from the Ca(OH)_2 with ethyl ether-alcohol (10:1) solution. The eluate was washed with water, dried over anhydrous Na_2SO_4 , and concen-

trated *in vacuo*. The principal maxima of this pigment in ethanol were at 445 and 472 $m\mu$. The concentration of xanthophylls was determined colorimetrically with an Evelyn colorimeter containing a No. 440 filter. Purified crystalline carotene (90% β - and 10% α -) in hexane was used as a standard for the colorimetric estimation. As determined by the Beckman spectrophotometer, model DU, the specific absorption coefficient of this carotene at 450 $m\mu$ was 242, which closely approximates the coefficient for lutein or zeaxanthin at the same wavelength (6). An aliquot of the xanthophylls was evaporated nearly to dryness *in vacuo* and was taken up in a definite weight of cottonseed oil which contained 1% α -tocopherol. Traces of ether were removed with reduced pressure and heat from a water bath at 50°C.

The final solutions were prepared so that they contained 65 μ g. or 130 μ g. of β -carotene equivalent, or these amounts of carotene plus 100, 300, or 600 μ g. of xanthophylls in 0.41 g. or 0.82 g., respectively, of cottonseed oil. In order that the chicks in the groups being compared receive identical amounts of β -carotene equivalent, a double-strength solution of the carotene was prepared. For the chicks receiving carotene, one part by weight of this solution was diluted with one part by weight of cottonseed oil. For the chicks receiving the xanthophylls, one part by weight of the double-strength carotene solution was diluted with one part by weight of double-strength xanthophyll solution.

TABLE I
Vitamin-A-low Diet

| | |
|---|------------------------------|
| Hegari (white grain sorghum) | 55 |
| Wheat middlings | 25 |
| Casein | 12 |
| Ca ₃ (PO ₄) ₂ | 2 |
| NaCl | 1 |
| Nonirradiated yeast | 5 |
| | <hr/> |
| MnSO ₄ ·2H ₂ O | 100 |
| Vitamin D (Nopdex) ^a | 20 mg. |
| | 200 A. O. A. C. ^b |
| | units |

^a Manufactured by Nopco Chemical Company, Harrison, N. J.

^b Association of Official Agricultural Chemists.

Two- to 3-day-old white leghorn chicks were wing-banded and housed in groups of 10-20 in electrically heated cages with raised screen bottoms. The chicks for each experiment were obtained from the same source and the same hatch. They were fed a vitamin-A-low diet (Table I) on which deficiency symptoms became apparent in 17-30 days. After the 14th day the chicks were weighed every 2 or 3 days until their weights declined or became constant. Three or four of them were killed and their livers analyzed for vitamin A to confirm vitamin A depletion. Then the chicks were placed upon experiment and the groups in each experiment equalized as to weights of the chicks. The carotene solutions or carotene-xanthophyll solutions were administered with a pipet daily except Sundays, for 14 feedings. In two experiments the daily

supplement was administered in three doses at 3-hr. intervals. On the 16th day the chicks were killed and the livers were removed, weighed, and placed in 12% alcoholic KOH. The vitamin A was determined by a method used previously (7).

DISCUSSION AND RESULTS

The results given in Table II show that when 65 μ g. of β -carotene equivalent were fed daily, neither 300 μ g. nor 600 μ g. of the xanthophyll concentrate affected the amount of vitamin A stored in the livers of the chickens. However, when 130 μ g. of carotene was fed daily, 100, 300, and 600 μ g. of xanthophyll significantly decreased the storage of vitamin A. Interestingly, this decrease was almost the same for all three levels, 32, 28, and 29%, respectively. It appears that the effect of xanthophylls depends, within limits, more upon the amount of carotene fed than upon the amount of xanthophylls fed.

Since the xanthophylls did not affect the 65- μ g. dosage of carotene it appeared likely that the 130 μ g. daily supplement of carotene would not be affected if it were given in small doses several hours apart. However, the results in Table III show that the xanthophylls exerted ap-

TABLE II
Effect of Xanthophylls Upon the Utilization of Carotene for Storage of Vitamin A in the Liver of the Chick

| Expt. no. | Daily supplement | | Number of chicks | Weight at start | Weight gained | Vitamin A in the liver | t Value and significance |
|-----------|------------------|--------------|------------------|-----------------|---------------|------------------------|--------------------------|
| | Carotene | Xanthophylls | | | | | |
| 1 | μ g. | μ g. | | $g.$ | $g.$ | μ g. | |
| | 65 | | 14 | 72 | 74 | 10.4 ± 1.2^a | 0.43 |
| | 65 | 300 | 15 | 73 | 71 | 9.8 ± 0.6 | Not significant |
| 2 | 65 | | 10 | 154 | 87 | 39.2 ± 4.5 | 0.77 |
| | 65 | 600 | 10 | 156 | 81 | 44.7 ± 5.5 | Not significant |
| 3 | 130 | | 12 | 108 | 98 | 171.5 ± 8.7 | 2.67 |
| | 130 | 100 | 15 | 108 | 92 | 117.0 ± 18.4 | $P < .02$ |
| 4 | 130 | | 19 | 73 | 66 | 71.4 ± 2.4 | 4.25 |
| | 130 | 300 | 19 | 73 | 71 | 51.4 ± 4.1 | $P < .01$ |
| 5 | 130 | | 11 | 97 | 64 | 91.4 ± 6.5 | 3.53 |
| | 130 | 600 | 14 | 98 | 65 | 64.8 ± 3.9 | $P < .01$ |

^a Standard deviation of the mean.

TABLE III
Effect of Xanthophylls Upon Carotene Utilization when Fed in Three Doses

| Daily supplement | | No. of doses | Number of chicks | Weight at start | Weight gained | Vitamin A in the liver | t Value and significance |
|------------------|----------------|--------------|------------------|-----------------|---------------|------------------------|--------------------------|
| Carotene | Xanthophylls | | | | | | |
| $\mu\text{g.}$ | $\mu\text{g.}$ | | | g. | g. | $\mu\text{g.}$ | |
| 130 | | 1 | 15 | 82 | 92 | 163.9 ± 10.8^a | 4.21 |
| 130 | | 3 | 8 | 84 | 82 | 270.0 ± 19.4 | $P < .01$ |
| 130 | 600 | 3 | 9 | 80 | 95 | 195.0 ± 17.3 | 2.88 |
| | | | | | | | $P < .02$ |

^a Standard deviation of the mean.

proximately the same inhibitory effect as for the single-dose feeding. It is to be noted also that the three-dose feeding gave significantly higher liver storages of vitamin A than the single-dose feeding.

The same level of carotene produced significantly different quantities of vitamin A in the livers of the different experimental groups of chicks. Whether or not this difference in storage is due to the difference in size of the chicks or to difference in lot cannot be determined from the data presented.

SUMMARY

Neither 300 $\mu\text{g.}$ nor 600 $\mu\text{g.}$ of xanthophylls reduced the storage of vitamin A in the livers of chicks fed 65 $\mu\text{g.}$ of β -carotene daily. However, 100, 300, and 600 $\mu\text{g.}$ of xanthophylls markedly reduced the storage of vitamin A when 130 $\mu\text{g.}$ of carotene was fed. This occurred even when the daily 130- $\mu\text{g.}$ supplement of carotene was given in three doses at intervals 3 hr. apart.

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Effects of Prolonged Exposure to Cold on the Thiamine Requirement of the Rat¹

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INTRODUCTION

Considerable data are available indicating that requirements for a number of nutrients are markedly increased under conditions of accelerated metabolism (1). This is particularly true for some of the B vitamins. An increased requirement for thiamine (2,3), pyridoxine (4), pantothenic acid (4), folic acid (5), and more recently vitamin B₁₂ (6-8) has been demonstrated following the administration of desiccated thyroid and other thyroactive substances. Requirements for B vitamins are also increased under conditions of prolonged physical exertion. The marked increase of metabolism during strenuous physical exertion (9) and the consequent ingestion of larger amounts of food both serve to increase body requirements for vitamin B₁ (10). Cowgill *et al.* (11) observed that the length of time required for dogs to develop anorexia on a vitamin B-deficient diet was one-third to one-half less in dogs permitted to exercise each day than in those who were not. Similar data have been reported by Guerrant and Dutcher (12) for the rat. Egana *et al.* (13), Johnson *et al.* (14), and others (15-17) found that severe physical exertion on a diet deficient in the B vitamins greatly hastened the onset of deficiency symptoms in man. Physical deterioration, accord-

¹ This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 310 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

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ing to Johnson *et al.* (14) occurred within 1 week and could be prevented or cured by the entire B complex containing 0.6 mg. of thiamine daily, but not by 2.0 mg. of thiamine hydrochloride alone, suggesting an increased requirement not only for thiamine hydrochloride but other B-complex factors as well. Prolonged exposure to cold with its accompanying increase in metabolic rate may also increase body requirements for certain nutrients. György (18) found that acrodynia induced by pyridoxine deficiency appeared significantly earlier in rats exposed to a low environmental temperature than in animals fed a similar diet at room temperature; while Dugal and Thérien (19) found that requirements for ascorbic acid in guinea pigs and rats were also increased under conditions of low environmental temperature. In the present communication, data are presented on the effects of prolonged exposure to cold on the thiamine requirement of the rat.

PROCEDURE AND RESULTS

The basal ration employed in the present experiment consisted of sucrose, 60.0%; casein,² 25.0%; salt mixture,³ 5.0%; and cottonseed oil (Wesson), 10.0%. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 20 mg.; riboflavin, 20 mg.; pyridoxine hydrochloride, 20 mg.; calcium pantothenate, 60 mg.; nicotinic acid, 60 mg.; ascorbic acid, 100 mg.; biotin, 5 mg.; folic acid, 10 mg.; *p*-aminobenzoic acid, 400 mg.; inositol, 800 mg.; vitamin B₁₂⁴, 60 µg.; 2-methyl-naphthoquinone, 10 mg.; and choline chloride, 2 g. Each rat also received once weekly 4.5 mg. of α -tocopherol acetate and a vitamin A-D concentrate containing 150 U. S. P. units of vitamin A and 15 U. S. P. units of vitamin D.⁵ Tests were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of $2 \pm 1.5^\circ\text{C}$. and (2) under standard laboratory conditions at an average temperature of approximately $23 \pm 2^\circ\text{C}$. One hundred and eighteen male rats of the University of Southern California strain were raised to maturity on a stock ration and were selected for the

² Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

³ Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

⁴ Vitamin B₁₂ Oral Grade Solids, Chas. Pfizer & Co., Brooklyn, New York: a vitamin B₁₂ concentrate containing 500 µg. vitamin B₁₂/g.

⁵ Nopco Fish Oil Concentrate, assaying 800,000 U. S. P. units of vitamin A/g. and 80,000 U. S. P. units of vitamin D/g.

present experiment at approximately 5 months of age and an average weight of 254 g. (range 222-318 g.). For a 10-day period prior to the start of the experiment all rats were fed the basal ration indicated above (30 rats at room temperature, the balance under cold-room conditions). Thirty-one of the 88 rats in the cold-room series died during this period. Of the remaining 57 animals 45 were selected for further study. These rats had lost an average of 27.4 g. during the 10-day adjustment period, mostly during the first 5 days of feeding. The remaining 12 animals in the cold-room series were discarded due to excessive loss in weight and poor condition and were not employed for subsequent study. The 30 rats in the room-temperature series gained an average of 14.7 g. during a similar period. Surviving animals in both the cold-room and room-temperature series were subsequently divided into three groups and fed the following diets: (a) basal ration (b) basal ration with thiamine hydrochloride omitted, and (c) basal ration with calcium pantothenate omitted. The groups consisted of 10 rats each in the room-temperature series and 15 animals/group in the cold-room series. Rats were placed in individual metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib.* the diets listed above. Animals were fed for 12 weeks or until death whichever occurred sooner.

Results are summarized in Table I. Data for the cold-room series were computed on the basis of the top 10 animals in each group in order to minimize variations in averages due to early deaths, infection, and atypical responses on the part of individual rats. Findings indicate that the length of survival of rats fed a thiamine-deficient diet was significantly decreased under conditions of low environmental temperature. One hundred per cent of the rats fed a thiamine-deficient diet succumbed during the course of the experiment with an average survival time of 27.6 days for the cold room and 64.7 days for the room temperature series. In contrast to the above, all rats fed the basal ration or the pantothenic-acid-deficient diet survived the experimental period of 12 weeks. Marked edema and erythema of the paws was observed in virtually all rats in the cold-room series. Progressive erythema and gangrene of the tail tip and edges of the ear was common, a finding previously reported by Gilson (20). Over half the animals in the cold room developed priapism with marked edema of the penis; and amputation of the entire tail was not uncommon. In general these findings occurred with equal frequency on all three dietary regimes. They did not occur in animals fed the same diets under room-temperature conditions. Food-consump-

TABLE I

Average Length of Survival of Rats Fed a Thiamine-Deficient Diet Under Cold-Room and Room-Temperature Conditions (10 Animals Per Group)

The values in parentheses indicate the number of animals which survived and on which averages are based.

| Dietary group | Initial body weight | Average body weight after the following weeks of feeding | | | Per cent surviving ^a | Length of survival of decedents ^b | |
|-------------------------|---------------------|--|----------------|----------------|---------------------------------|--|------------------------|
| | | 4th | 8th | 12th | | Average | Range |
| | | Cold-room series | | | | | |
| Thiamine-deficient | <i>g.</i> 231.6 | <i>g.</i> 155.7 (4) | <i>g.</i> — | <i>g.</i> — | 0 | <i>days</i> 27.6 ± 1.1 | <i>days</i> (24-32) |
| Pantothenate-deficient | 226.5 | 211.0 (10) | 210.5 (10) | 203.8 (10) | 100 | — | |
| Basal ration | 228.6 | 225.1 (10) | 229.4 (10) | 237.6 (10) | 100 | — | |
| Room-temperature series | | | | | | | |
| Thiamine-deficient | 266.7 | 301.4 (10) | 188.3 (8) | — | 0 | 64.7 ± 2.6 | (54-78) |
| Pantothenate-deficient | 264.8 | 306.3 (10) | 298.0 (10) | 283.3 (10) | 100 | — | |
| Basal ration | 262.4 | 309.7 (10) | 330.1 (10) | 336.5 (10) | 100 | — | |

^a Experimental period, 84 days.

^b Including standard error of the sample mean calculated as follows: $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$

where d is the deviation from the mean and n is the number of observations.

tion determinations during the first week of feeding indicate that during this period caloric intake under cold-room conditions was approximately 25% greater on each of the three diets tested than under room-temperature conditions. Food consumption was not measured subsequent to this period. It was apparent, however, from gross examination of the food cups that rats fed the thiamine-deficient diet developed anorexia during the fourth week of feeding under cold-room conditions and during the seventh week of feeding in the room-temperature series.

It is well established that the metabolic rate of rats is markedly

increased on prolonged exposure to cold (21,22). A significant increase in caloric intake also occurs under conditions of low environmental temperature. Both factors (*i.e.*, increased metabolic rate and increased caloric intake) augment the thiamine requirement of the rat (11). These factors appear to be responsible, at least in part, for an accelerated rate of depletion in rats fed a thiamine-deficient diet at low environmental temperatures under conditions of the present experiment.

Present findings indicate that prolonged exposure to cold has little if any effect on the pantothenic acid requirement of the rat as measured by gross appearance and length of survival on a pantothenic-acid-deficient diet. The effects of feeding such a ration were investigated in the present experiment in view of findings by Ashburn (23) and others that diets deficient in pantothenic acid lead to cellular atrophy, necrosis, and other pathological effects in the adrenal cortex of the rat. Since a deficiency of adrenal cortical hormones impairs the rats' ability to adjust to low environmental temperatures (24), it was felt that if requirements for pantothenic acid were materially increased following prolonged exposure to cold and if pantothenic acid deficiency impairs the production of adrenal cortical hormones, then rats fed a diet deficient in this vitamin would succumb more rapidly under cold room than room temperature conditions. With the exception of a slight loss in body weight, however, no evidence of pantothenic acid deficiency was observed in rats fed a diet deficient in this factor either under cold-room or room-temperature conditions during an experimental period of 12 weeks.

SUMMARY

Adult male rats were fed a purified ration deficient in thiamine hydrochloride and their average survival time was determined under cold-room (2°C.) and room-temperature (23°C.) conditions. Length of survival was significantly decreased at the lower environmental temperature averaging 27.6 days for the cold-room and 64.7 days for the room-temperature series.

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Properties of Dog Gastric Urease¹

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INTRODUCTION

In previous investigations gastric urease was studied with respect to acid secretion in patients with gastric cancer and duodenal ulcer (1), and evidence was subsequently obtained to support the possibility that urease, by releasing ammonia, plays a part in regulating gastric acidity and in protecting the mucosa against the action of acid pepsin (2). While the properties of soybean and jack bean urease have been investigated extensively, relatively little is known concerning the animal enzyme. Luck and Seth (3) reported that, with respect to optimum pH and specificity, gastric urease and that of the soybean act in the same manner. The effect of pH and temperature on the gastric enzyme was investigated by Fossel (4), and he also reported that cupric and cobaltous ions inhibited the urease while cyanide did not.

Earlier work on inhibitors of the plant urease was reviewed by Mann and Mann (5), who found that exposure of the gastric mucosa to solutions of cupric or mercuric salts, quinone, brilliant green, or crystal violet inhibited acid secretion in dogs. These authors pointed out that while the effects on gastric acidity may be based on inactivation of the urease by the compounds, the possibility remains that the effect resulted from inactivation of other enzymes. No data were presented on the influence of the compounds on gastric urease itself.

The present study was instituted to obtain data relative to the effects of mercapto-binding compounds, heavy metal cations, dyes, and other substances on dog gastric urease. No purification of the enzyme was attempted since it was considered advantageous for this study to employ the urease in its natural milieu.

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EXPERIMENTAL

Enzyme Preparation

The body portions of dog stomachs were excised from the animals anesthetized with nembutal.² The mucosa was scraped off, ground in a mortar with washed and ignited sand, extracted in a refrigerator overnight with 10–20 ml. of 30% glycerol/g. of scrapings, and then centrifuged for 15 min. at 2500 r.p.m. ($1180 \times g$). The supernatant was employed as the source of enzyme, and the portion not required immediately was stored in the frozen state in 10-ml. tubes. Freezing and thawing did not affect the urease activity.

Procedure

The standard procedure used was to add 0.5 ml. of the enzyme solution to 1 ml. of an aqueous solution of the compound to be tested or water, and after 5 min. at room temperature 1 ml. of buffered substrate was added. The digestion was allowed to proceed for 1 hr. at 37.5° , after which the ammonia in a 2-ml. portion was determined by placing the sample in the outer well of a Conway cup (6), 7 cm. in diameter, with 2 ml. of 0.1 *N* H_2SO_4 in the center well, adding 1 ml. of 2.5 *N* NaOH to the sample, sealing the cup with a vaselined groundglass plate and allowing the ammonia to diffuse for 3 hr. at room temperature. The liquid in the center well was quantitatively transferred to a graduated test tube by rinsings with distilled water, and the volume was made up to 15 ml. To 5-ml. aliquots, 4 ml. of water and 1 ml. of Nessler's reagent were added and colorimetry was carried out by comparison with standards of known ammonia concentration. Control experiments were included in which either the enzyme or substrate was omitted from the solution in which they were normally present.

Digestion Period, Buffers, Enzyme, and Substrate Concentration

Preliminary experiments established that a linear relationship was maintained between enzyme activity and time over the period tested (3 hr.) using 0.5 ml. of the enzyme solution and 1 ml. of 3% urea in either phosphate or maleate buffer. Then, using a 1-hr. digestion period, linearity was observed between activity and volume of enzyme solution over the range examined (0–0.8 ml.). Comparison of phosphate buffer (1 vol. 0.3 *M* KH_2PO_4 + 1 vol. 0.15 *M* NaOH) with maleate buffer (1 vol. 1.0 *M* maleic acid + 1 vol. 1.77 *M* NaOH), both at pH 6.8, revealed a slightly greater activity with the maleate. Harmon and Niemann (7) reported that phosphate inhibits jack bean urease. Finally, the effect of substrate concentration was studied using the 1-hr. digestion and 0.5 ml. of enzyme preparation (Fig. 1). The optimum urea concentration of 3.0 *M* (18%) is surprisingly high, but it is undoubtedly conditioned by the concomitant substances in the crude enzyme material used. Activity-pS curves showed no correspondence with those following the Michaelis-Menten theory, the greatest deviations being observed at the lowest urea concentrations. Impurities in the enzyme solution may well be responsible for this behavior too.

² The authors are grateful to Dr. D. J. Ferguson for this material.

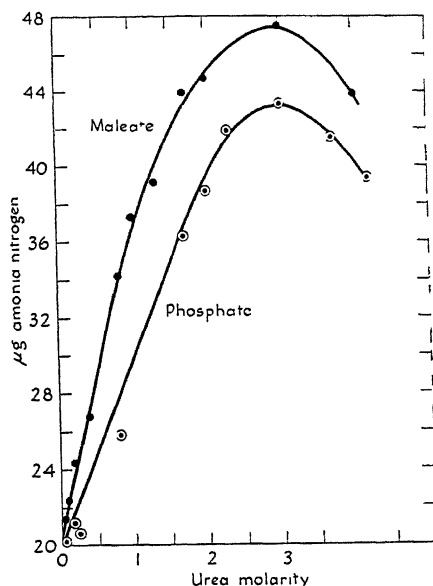


Fig. 1. Effect of urea concentration on urease activity.

From the foregoing, the optimum conditions for the urease measurement were established. The 1 ml. of buffered substrate used in the reaction mixture consisted of 45% urea in the maleate buffer.

TABLE I

Effect of Certain Drugs on the Activity of Dog Stomach Urease

| Drug (final conc 0.25%) | Change in activity per cent |
|---|--------------------------------|
| Sodium salicylate | + 8 |
| Atropine sulfate | +14 |
| Enterogastrone ^a | 0 |
| Benadryl | +27 |
| Histamine dihydrochloride | - 8 |
| Pilocarpine nitrate | - 1 |
| Physostigmine sulfate | - 5 |
| Acetyl- β -methylcholine chloride | + 1 |
| Carbaminoyl choline chloride | - 2 |
| Gastric mucin (Stearns) (final conc. 0.4%) | + 6 |
| Sodium fluoride | -96 |

^a The authors are indebted to Dr. H. F. Hailman, The Upjohn Co., Kalamazoo, Mich., for this drug.

RESULTS AND DISCUSSION

Although a functional relationship seems to obtain between the gastric acid secretory response to histamine and urease activity in pathological conditions (1), it appears from Table I that drugs which have an influence on the acid secretion have no appreciable effect *in vitro* on the dog stomach urease. The fluoride, as an ion of known inhibiting properties, was included for comparison. If these drugs can influence the urease activity *in vivo*, they must do so by means of a physiological mechanism rather than by direct action on the enzyme. The *in vivo* effect is now under study.

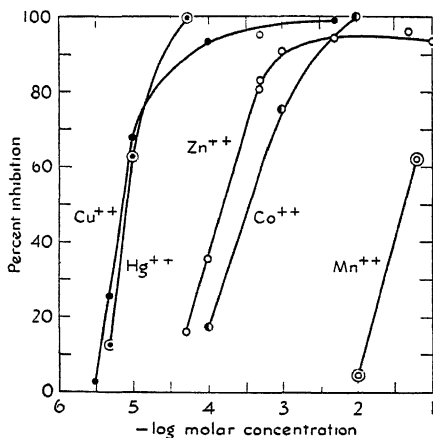


FIG. 2. Inhibition of urease by heavy metal cations.

The report of Fossel (4) that cupric and cobaltous ions inhibit gastric urease is born out by the data in Fig. 2 which, however, include the effects of a range of ion concentrations. Mercuric, zinc, and manganous ions were also included in this study since Mann and Mann (5) had found that, while the mercuric ion inhibited acid secretion when applied to the gastric mucosa, the other two ions were without effect. From Fig. 2 it is apparent that all of these ions can inhibit the urease although the concentrations required differ considerably in some cases.

The work of Hellerman *et al.* (8,9) on the mercapto nature of crystalline plant urease led the authors to investigate the mercapto properties of the gastric enzyme. The first three mercapto-binding compounds in

Table II proved to be powerful inhibitors and so did iodoacetic acid (Fig. 3). These facts, together with the observation that cysteine and glutathione activate the enzyme (Fig. 3), and that heavy metal ions inhibit, make it clear that the gastric urease is also a mercapto enzyme.

Sulfanilamide, which is an inhibitor of carbonic anhydrase, and azide, which is also an inhibitor of carbonic anhydrase as well as of iron-containing oxidative enzymes (10), had little effect on the urease (Table II) in concentrations that give marked inhibition with the other^a en-

TABLE II
Effect of Other Compounds on the Activity of Dog Stomach Urease

| Compound | Final conc. (per cent saturation, 23°) | Change in activity per cent |
|--|--|--------------------------------|
| Phenylmercurinitrate | 0.4 | -100 |
| | 0.3 | - 82 |
| | 0.2 | - 39 |
| | 0.1 | - 23 |
| | 0.04 | - 1 |
| <i>o</i> -Iodosobenzoic acid ^a | 40 | - 87 |
| | 20 | - 74 |
| | 4 | - 38 |
| | 0.4 | - 1 |
| <i>p</i> -Chloromercuribenzoic acid ^a | 16 | -100 |
| | 12 | - 62 |
| | 8 | - 24 |
| | 4 | - 10 |
| Sulfanilamide | 0.02 ^b | + 3 |
| Sodium azide | 0.2 ^b | + 6 |

^a The authors are indebted to Dr. L. Hellerman, Johns Hopkins University, School of Medicine, for these compounds.

^b Concentration expressed is molar.

zymes. This differential action is of interest since carbonic anhydrase is probably involved in acid secretion.

Cyanide had some activating effect (Fig. 3) as might be expected for a mercapto enzyme, while ascorbic acid was strongly inhibitory. The latter action may be due to the effect of oxidized ascorbic acid. Traces of copper or iron in the water and enzyme preparation could account for such oxidation.

Mann and Mann (5) had shown that brilliant green stains the gastric mucosa and inhibits its acid secretion, while Congo red does neither,

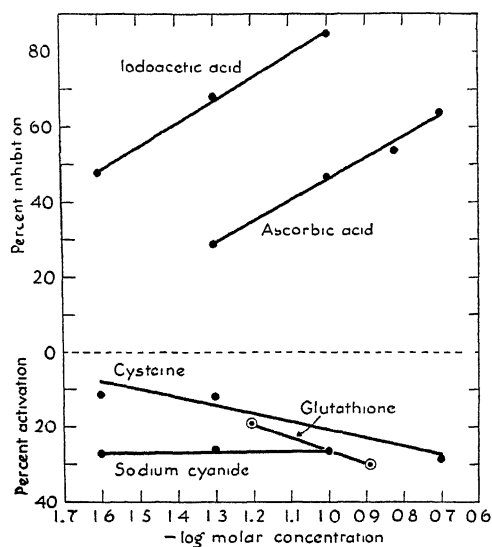


FIG. 3. Effect of mercapto, mercapto-binding, and other compounds on urease activity.

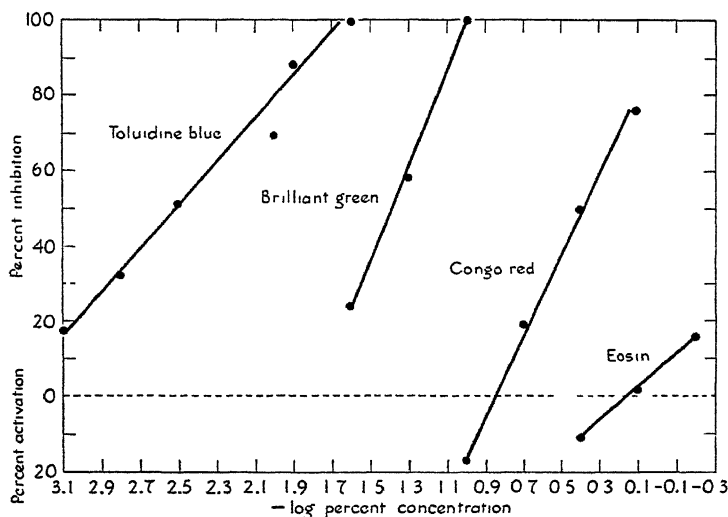


FIG. 4. Effect of dyes on urease activity.

and Quastel (11) found that triphenylmethane dyes such as brilliant green are effective inhibitors of plant urease. From Fig. 4 it may be seen that both brilliant green and Congo red are capable of inhibiting the gastric urease, although the former is efficacious in lower concentrations. It is also apparent that toluidine blue is even more effective than brilliant green, and that eosin (yellowish) is practically without influence. The fact that the inhibiting potency is greatest with the basic dyes and least or nonexistent with the acid dyes may indicate that some essential group in the urease is acid.

In this connection it is interesting to recall the conclusion of Visscher (12) that, from the available evidence, only those dyes whose chromogen is in the cation can be secreted in gastric juice. Thus Congo red and eosin are not secreted while toluidine blue and brilliant green are. Whether this can be related to the present findings remains to be seen.

SUMMARY

A variety of drugs which either inhibit or potentiate gastric secretion had no significant effect *in vitro* on dog stomach urease.

The range of concentrations in which cupric, mercuric, zinc, cobaltous, and manganous ions inhibit the gastric urease was determined.

The mercapto nature of the enzyme was demonstrated by its inhibition by mercapto-binding compounds, and its activation by cysteine and glutathione. Cyanide was found to have some activating effect, sulfanilamide and azide were practically without influence, and ascorbic acid was strongly inhibitory under the conditions employed.

Toluidine blue, brilliant green, Congo red, and eosin were shown to inhibit the gastric urease in the order named, eosin having only a slight effect. This may be taken to indicate the acidic nature of some essential part of the enzyme.

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LETTER TO THE EDITORS

The Electrophoretic and Biological Complexity of Ovomucin¹

The inhibitory activity of egg white (1) against hemagglutination by influenza viruses is associated closely with a highly viscous, fibrillar, macromolecular carbohydrate-protein complex with the solubility properties of ovomucin (ovomucoid) (2-4). Several widely different methods of purification, based on (a) solubility (3), (b) sedimentation (3), or (c) filtration (5) properties, have each yielded preparations with specific activities (activity/N ratios) 50-70 times the specific activity of crude egg white. Further purification by these methods has thus far proved impossible. Although only a single sedimentable component (sedimentation constant about 30 S) can be demonstrated in these preparations (3), electrophoresis usually reveals the presence of more than one component (3,6).

The present studies have confirmed the previous findings and established the presence of three components with mobilities of -3.5 , -6.7 , and -10.0×10^{-6} cm.² sec.⁻¹ volt⁻¹ in phosphate buffer of pH 7.2 and ionic strength 0.1. The -3.5 component contributes roughly 60-90% of the total refractive increment, the partition of the remainder between the two faster components being dependent on the mode of preparation and the subsequent treatment of the material.

It has been found that both the -6.7 and the -10.0 components possess inhibitory activity, while the -3.5 component is essentially inert. Under some conditions (*e.g.*, on dilution) the -6.7 component appears to dissociate, yielding additional activity with mobility -10.0 . A fraction devoid of the -3.5 component had a specific activity about 190 times that of egg white, a finding which accords with the electrophoretic composition of the particular preparation of semipurified inhibitor employed. Electrophoretic fractions greatly enriched with respect to the inert or to the active components each possessed an extremely high viscosity increment; however, purified swine influenza virus was able to reduce the viscosity [cf. (2)] only of the fraction enriched with respect to the active components. Moreover, each fraction showed a single sedimenting boundary, with a sedimentation constant experimentally identical with that of the single boundary observed with the unfractionated mixture as well as with that of the inhibitory activity of active preparations. Finally, the active and inert components are all retained by ordinary filter papers under suitable conditions; and analyses for carbohydrate (orcinol) and hexosamine support the conclusion that all are mucoproteins.

While it has not yet been possible to isolate the -6.7 component for direct study, it would appear that the three components are extremely similar in their physical

¹ This work was supported by a research grant from the National Cancer Institute, U. S. Public Health Service, and by the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

properties, except for charge density, and that together they comprise the ovomucin of egg white. This view provides a reasonable explanation for the limit of purification attained by methods which did not include electrophoresis, since this limit is in fair accord with the ovomucin content of egg white. The newer data, while substantiating the previous conclusion (3,4) that inhibitory activity is a property of ovomucin, indicate that the concept of ovomucin as a single mucoprotein entity is no longer tenable. Thus far, the components of ovomucin have been distinguished sharply only by electrophoresis; however, there is some evidence that they differ also in properties like solubility, filterability, heat stability, and viscosity.

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Book Reviews

The Hormones. Vol. II. Edited by GREGORY PINCUS and KENNETH V. THIMANN. Academic Press Inc., New York, N. Y., 1950. ix + 782 pp. Price \$12.50.

This is the concluding volume of a reference work covering the chemistry, physiology, and applications of hormones. The first volume appeared in 1948 and was reviewed in this Journal [22, 160 (1949)]. There has been no studied attempt on the part of the editors to segregate chemical and physiological aspects of the subject into the separate volumes. However, the volume under review consists largely of physiological material.

The plan of this volume, like that of the first, is a collection of monographs as chapters which are contributed by experienced workers in the various specialties. Several of the monographs are supplementary to related chemical and biochemical treatment of topics covered in the first volume, in some instances by the same authors. In this category are the chapters on "The Physiology of Ovarian Hormones" by Pincus, "Physiology of Androgens" by Ralph Dorfman, "Physiology of the Gonadotrophins" by Evans and Simpson, and "The Hypophysis and Diabetes Mellitus" by Bennett and Evans. An exhaustive summation of the present status of our knowledge of the "Physiology of the Adrenal Cortex" is contributed by Noble. Two of the endocrine glands not touched upon in volume I are covered in "The Chemistry and Physiology of the Thyroid Hormone" by Salter, and "Hormones of the Posterior Pituitary" by Waring and Landgrebe.

A departure from the usual arrangement in a reference work on hormones is the inclusion of three chapters entitled "Chemical Control of Nervous Activity," A. "Acetylcholine" by Nachmansohn; B. "Adrenalin and Sympathin" by Blaschko; and C. "Neurohormones in Lower Vertebrates" by Parker. Each monograph is excellent in its own way, but collectively, they make rather uneven reading. The chapter on acetylcholine deals primarily with the role of acetylcholine and its esterase in the chemical mechanics of conduction of the nerve impulse, whereas, the chapter on adrenaline and sympathin is more concerned with the biochemistry of chromaffin tissue and the intermediary metabolism of adrenaline and noradrenaline.

The final chapter is a discussion on "Clinical Endocrinology" by Freeman. In 40 pages the author covers the pituitary, thyroid, parathyroid, testis, ovary, and adrenal glands. This is a well-written monograph but its extreme brevity makes one wonder if the editors should not have considered adding a third volume to their work. It might deal *in extenso* with past and potential applications of animal hormones in both human and veterinary medicine as well as with those of phytohormones in horticulture.

The volume has its own author and subject index. With Volume I it constitutes the most thorough, authoritative and up-to-date treatise at present available on the chemistry and physiology of hormones.

JOSEPH J. PFIFFNER, Detroit, Michigan

Quantitative Chemical Analysis. By GEORGE L. CLARK, Professor of Chemistry and Head of the Division of Analytical Chemistry, University of Illinois, Urbana, Ill., LEONARD K. NASH, Assistant Professor of Chemistry, Harvard University, Cambridge, Mass., and ROBERT B. FISHER, Assistant Professor of Chemistry, Indiana University, Bloomington, Indiana. W. B. Saunders Co., Philadelphia and London, 1949. v + 448 pp. Price \$4.25.

According to the opinion of this reviewer, the above new book on Quantitative Chemical Analysis is one of the best that has been published in this field in a long time. This text utilizes the experience gained in teaching the subject for over twenty-five years. The arrangement of subject matter is excellent and yet elastic enough to enable one to use this book for different types of courses in quantitative analysis.

This book starts with seventeen pages of a review of fundamental principles of Chemistry and finishes with fifteen pages of essential mathematics for Quantitative Analysis. These two sections are frequently omitted in a Quantitative Analysis text even though they are a great help to a beginning student. The text is divided into three main sections: Gravimetric Analysis, Volumetric Analysis, and Optical Analysis.

The theory of gravimetric and volumetric analysis is very well written and accompanied by an excellent selection of experimental procedures. The chapter on purity of precipitates illustrates by means of actual electron photo-micrographs many of the properties of crystalline precipitates, adsorption, and effects of impurities. This visual method is much more striking and effective than theoretical discussion.

This third section consists of two chapters of twenty-two pages. The first chapter deals with "Colorimetry and Spectrophotometry," and the second, entitled "Other Optical and Electrical Methods of Analysis," discusses turbidimetry, nephelometry, fluorimetry, electrodeposition methods, potentiometric titrations, conductometric titrations, polarography, emission spectrographic methods, X-ray methods, and physical-constant methods. This chapter ends with a fine list of selected references on instrumental analysis.

The authors also introduce to the student the statistical treatment of experimental data which from the viewpoint of both student and instructor is a very useful addition.

The appendix, besides treating of the before mentioned essential mathematics for Quantitative Analysis and statistical treatment of experimental data, also contains notes on a method of standardizing weights, electromotive series of the metals, table of solubility products, ionization constants of acids and bases, complex-ion instability constants, specific volume of water, a 1949 atomic-weight table, and, finally, methods for the preparation of some fifty different indicator solutions. The two end sheets of the text where one usually finds atomic weight tables or logarithm tables is a table of indicator color changes with pH. Even though calculations using logarithms are discussed fully, the authors have omitted the inclusion of a logarithm table.

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The Mode of Action of the Antibiotic, Usnic Acid ¹

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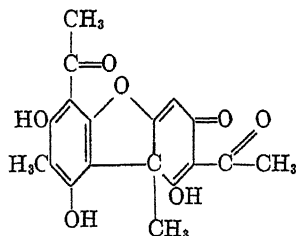
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INTRODUCTION

The ability of antibiotics to inhibit the growth of microorganisms has led to wide applications although little was understood about their actions. Recent studies have disclosed the mode of action of some of these compounds although details of the mechanism involved remain to be elucidated. Atabrin (1), gramicidin (2), and aureomycin (3) have been found to inhibit oxidative phosphorylations without depressing respiration. In this respect their physiological effect is similar to that of dinitrophenol (4). Usnic acid,² a natural product of lichen species (5), has been shown to exert antibacterial action, especially toward gram-positive organisms (5-8). It also has been shown by Marshak and Harting (9) to inhibit phosphate uptake and cleavage of *Arbacia* eggs without depressing respiration. This report describes enzyme studies in which it was found that usnic acid has a mode of action similar to that

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² Usnic acid has the following structure [CURD, F. H., AND ROBERTSON, A., *J. Chem. Soc.* **1938**, 894; SCHOPF, C., AND ROSS, F., *Ann.* **546**, 1 (1941)]:



of gramicidin, atabrin and aureomycin in that it "uncouples" the fixation of inorganic phosphate which is normally associated with tissue oxidative reactions.

EXPERIMENTAL

The twice-washed residues of rat kidney or liver homogenate were prepared as described elsewhere (10). The kidney and liver residue were taken up in sufficient cold isotonic KCl to give 30% and 50% suspensions, respectively. The final kidney residue suspension was analyzed for nitrogen by a micro direct-Nesslerization procedure. Oxygen consumption was measured by conventional Warburg manometry for 60 min. at 37°C. and is expressed in terms of 1.0 mg. nitrogen. The composition of the reaction mixture is presented with the experimental results.

The manipulation of flasks described elsewhere (10) was followed in the oxidative phosphorylation experiments with rat liver washed residue. The 10-min. oxygen consumption and phosphorus uptake are recorded without calculation to nitrogen content. Each experiment included a zero-time flask to obtain the original concentration of inorganic P, a control flask, and experimental flasks which contained usnic acid. The aqueous solution of usnic acid was prepared by adding 2 *N* KOH in small increments (0.01 ml.) to the suspension of usnic acid crystals until the pH was 7.0-7.5. The resulting solution was a very pale yellow.

RESULTS

Oxidation of Krebs' Cycle Intermediates

All the compounds used were vigorously oxidized by the washed residue of rat kidney. Each substrate was studied in a separate experiment. Results are shown in Table I. With 10^{-6} *M* usnic acid, stimulation of oxygen consumption was observed with glutamate, α -ketoglutarate, *cis*-aconitate, and pyruvate-fumarate. The oxidation of citrate, succinate, and malate was initially stimulated but depression soon developed so that over-all O₂ consumption for 1 hr. was less than in the control flasks. The slow oxidation of pyruvate alone was not stimulated whereas the oxidation of fumarate alone was depressed throughout the experimental period. Marked inhibition was observed with all substrates when the concentration of usnic acid was increased; 10^{-4} *M* usnic acid depressed respiration 50-90%.

Effect of Usnic Acid on Hexokinase

Before proceeding with studies of oxidative phosphorylation it was necessary to determine whether usnic acid had any effect on hexokinase since this enzyme is used to transfer phosphate from the liver homog-

TABLE I

Effect of Usnic Acid on the Respiration of Washed Rat Kidney Particles

Each Warburg vessel contained 30 μ moles of substrate. In addition, each flask contained 2×10^{-3} M adenosine triphosphate (ATP), 1.7×10^{-2} M phosphate buffer pH 7.2, 3.3×10^{-3} M MgSO_4 , 1.25×10^{-5} M cytochrome c, 0.2 ml. of a 30% suspension of a washed residue of rat kidney, and 0.15 M KCl to 3.0 ml., $t = 37^\circ\text{C}$.

| Substrate | Control | $Q_{O_2}(N)^a$ | | | | |
|-------------------------|---------|-----------------------------|-----|-----|-----|-----|
| | | Usnic acid, $M \times 10^6$ | | | | |
| | | 1 | 10 | 50 | 100 | 200 |
| Glutamate | 364 | 431 | 77 | 95 | 69 | 16 |
| | 429 | 488 | 102 | | 82 | |
| | 417 | 482 | 119 | | 52 | |
| | 345 | 365 | 60 | | 32 | |
| | 350 | 373 | 61 | | 41 | |
| α -Ketoglutarate | 457 | 542 | 176 | 212 | 141 | |
| Succinate | 330 | 230 | 155 | 100 | 87 | |
| Fumarate | 252 | 198 | 77 | 78 | 71 | |
| Malate | 179 | 184 | 58 | 77 | 82 | |
| Pyruvate-fumarate | 390 | 425 | 58 | 109 | 59 | |
| Citrate | 265 | 195 | 32 | 22 | 21 | |
| cis-Aconitate | 428 | 496 | 112 | 89 | 61 | |

^a $Q_{O_2}(N) = \text{cu. mm. } O_2 \text{ consumed/mg. tissue nitrogen/hr.}$

enate system to glucose. As shown in Table II, usnic acid at the concentrations used in the respiration experiments, did not inhibit yeast hexokinase. The hexokinase used was the fraction obtained at Step 3a in the isolation procedure of Berger *et al.* (11).

TABLE II

Yeast Hexokinase Activity in the Presence of Usnic Acid

Side arm: 8×10^{-3} M ATP, 6.6×10^{-3} M MgCl_2 , 3.3×10^{-3} M NaHCO_3 . Main compartment: 2.4×10^{-2} M NaHCO_3 , 3.3×10^{-2} M glucose, 0.05 ml. hexokinase preparation, H_2O to give a final volume of 3.0 ml. Gaseous phase = 95% N_2 :5% CO_2 ; $t = 30^\circ\text{C}$.

| | Usnic acid, $M \times 10^6$ | | | |
|---|-----------------------------|-----|-----|-----|
| | 0 | 2 | 8 | 32 |
| $\mu\text{l. CO}_2$ produced in 10 min. | 185 | 174 | 192 | 195 |

Oxidative Phosphorylation

The effect of usnic acid upon oxidative phosphorylation with pyruvate as substrate is shown in Fig. 1. The addition of very small quantities of usnic acid ($1 \times 10^{-6} M$) increased (very slightly) the amount of phosphate taken up in each of the three experiments without influencing oxygen uptake. As the concentration of usnic acid was increased, progressive inhibition of phosphate uptake occurred without marked

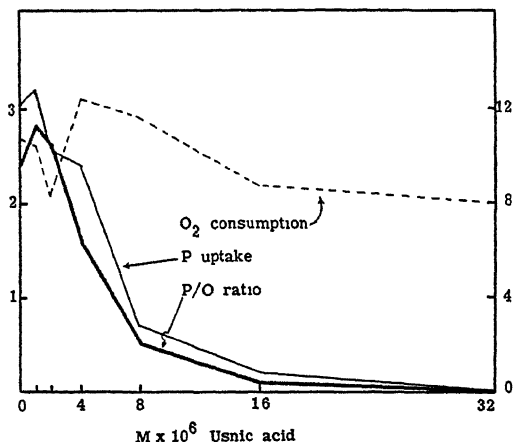


Fig. 1. Effect of usnic acid on pyruvate oxidation and fixation of inorganic phosphate. Right ordinate: $\mu\text{moles—P}$ esterified; left ordinate: μmoles oxygen consumed and P/O ratio. Each flask contained $0.0067 M$ $MgCl_2$, $1.3 \times 10^{-5} M$ cytochrome c, $0.0067 M$ potassium phosphate (pH 7.3), $0.002 M$ adenosine triphosphate (ATP), $0.01 M$ KF, $0.017 M$ sodium pyruvate, $2.0 \times 10^{-5} M$ potassium fumarate, 0.4 ml. of the 50% suspension of washed residue of rat liver homogenate and $0.154 M$ KCl to 3.0 ml.; 0.1 ml. of $0.3 M$ glucose and 0.05 ml. of hexokinase preparation were tipped in from side arms at zero time. KOH in center well to absorb CO_2 ; $t = 30^\circ C$. The data are averages of three similar experiments.

depression of oxygen consumption. In these experiments with liver homogenates, vigorous respiration occurred in the presence of concentrations of usnic acid which were shown to be strongly inhibitory in Table I. The following factors may have been responsible for this difference: more washed residue was used, the washed residue of liver may tolerate greater concentrations of usnic acid than does washed kidney residue, the experiments were carried out at a lower temperature, and

TABLE III

*Oxidative Phosphorylation with α -Ketoglutarate as Substrate*Experimental conditions as in Fig. 1 except that the substrate was α -ketoglutarate at 0.017 M.

| | Control | Usnic acid, $M \times 10^6$ | | | | | |
|--------------------------------------|---------|-----------------------------|------|------|------|-----|-----|
| | | 1 | 2 | 4 | 8 | 16 | 32 |
| O ₂ consumed, μ moles | 4.1 | 3.9 | 4.0 | 4.2 | 4.6 | 3.2 | 1.8 |
| P uptake, μ moles | 16.6 | 17.0 | 16.3 | 16.1 | 14.9 | 3.4 | 0 |
| P/O ratio | 2.0 | 2.2 | 2.0 | 1.9 | 1.6 | 0.5 | 0 |

the experimental period was shorter. With 32×10^{-6} M usnic acid, no phosphorus uptake was obtained and the recoveries of inorganic P were greater than present in the zero-time flask. This observation is similar to findings with high concentrations of dinitrophenol (12) which discharge P from the tissue preparation.

TABLE IV

Effect of Usnic Acid on Oxidative Phosphorylation with Various Substrates

Experimental conditions described under Fig. 1

| | Control | | | Experimental Usnic acid (8×10^{-6} M) | | |
|--|----------------------------|-------------|-----|--|-------------|------------------|
| | O ₂ Consumed | P Uptake | P/O | O ₂ Consumed | P Uptake | P/O |
| Pyruvate, 50 μ moles plus fumarate, 0.87 μ moles | μ moles | μ moles | | μ moles | μ moles | |
| | 4.3 | 17.9 | 2.1 | 3.8 | 4.0 | 0.5 |
| | | | | 3.4 | 8.8 | 1.3 ^a |
| | 3.5 | 19.1 | 2.7 | 3.5 | 2.9 | 0.4 ^b |
| Citrate, 60 μ moles Isocitrate, 60 μ moles α -Ketoglutarate, 50 μ moles Glutamate, 60 μ moles Succinate, 60 μ moles Fumarate, 50 μ moles Malate, 50 μ moles | | | | 3.2 | 0.4 | 0 ^c |
| | 5.5 | 25.2 | 2.3 | 4.5 | 9.0 | 1.0 |
| | 3.5 | 15.1 | 2.1 | 4.0 | 8.4 | 1.0 |
| | 3.7 | 20.1 | 2.7 | 2.1 | 5.9 | 1.4 |
| | 4.5 | 21.4 | 2.4 | 4.6 | 15.8 | 1.7 |
| | 4.9 | 14.6 | 1.5 | 4.7 | 8.8 | 0.9 |
| | 2.1 | 13.2 | 3.1 | 2.1 | 6.7 | 1.6 |
| | 2.0 | 11.9 | 3.0 | 1.8 | 3.2 | 0.8 |

^a 1×10^{-5} M dinitrophenol.^b 2×10^{-5} M dinitrophenol.^c 3×10^{-5} M dinitrophenol.

It was of interest to investigate the action of this antibiotic upon the utilization of other metabolites. Table III shows results obtained with varying concentrations of usnic acid when α -ketoglutarate was the substrate. The results are similar to those obtained with pyruvate except that somewhat higher concentrations of usnic acid were required to obtain the same degree of "uncoupling."

The results of experiments with 8×10^{-6} *M* usnic acid and several other substrates are shown in Table IV. In each experiment there was marked inhibition of phosphorylation although the effect on O_2 consumption was variable. The oxidation of citrate, succinate, and malate was depressed as noted in the 1-hr. respiration experiments (Table I). Also shown are results with dinitrophenol which indicate that these two compounds exert their action at approximately the same concentration.

DISCUSSION

Barry (6), Stoll (7), and Marshak (8) observed that usnic acid at 2 $\mu\text{g./ml.}$ inhibited the growth of human tubercle bacilli and of certain gram-positive organisms. In the experiments described here, usnic acid, at 1.3–2.6 $\mu\text{g./ml.}$, was found to "uncouple" the oxidative phosphorylations of a washed residue of rat liver homogenate. The similarity in effective concentrations in these two cases suggests that the effect on phosphorylation may be responsible for the antibiotic action. Relatively high concentrations (100 $\mu\text{g./ml.}$) of usnic acid were required to inhibit P uptake and cleavage in *Arbacia* eggs (9). Likewise, relatively large doses were tolerated by mice (5). This suggests that animal cells are less readily penetrated by usnic acid, thus keeping the concentration below that found inhibitory to energy-coupling mechanisms in these experiments. The enzymes of the washed liver residue were probably freely accessible to the antibiotic, thus the lower concentration was effective. Since gram-positive organisms succumbed to this same low concentration of usnic acid it would appear that they are readily penetrated by this antibiotic. Differential permeability of bacterial and host cells to certain antibiotics may be the basis for selective action of these agents and hence for their therapeutic usefulness.

ACKNOWLEDGMENT

We are indebted to Dr. Alfred Marshak for a generous supply of usnic acid.

SUMMARY

The antibiotic, usnic acid, was found to prevent the uptake of orthophosphate which is normally associated with the oxidation of various substrates by a washed residue of rat liver homogenate. In this respect its biological activity resembles that of certain other antibiotics and of dinitrophenol.

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Deposition of Radioactive Calcium in Rachitic and Nonrachitic Chick Tibia from Oral and Intramuscular Doses of Ca^{45}

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INTRODUCTION

The use of Ca^{45} in the study of calcium metabolism and vitamin D action is limited by the fact that Ca^{45} is available only with a low specific activity. Although larger than trace quantities must be employed some investigations lend themselves to the use of Ca^{45} in its present form.

Migicovsky and Emslie (1948) showed that under starvation conditions administration of vitamin D decreased the excretion of calcium, and when chicks had Ca^{45} deposited in the skeleton 5 days before vitamin D treatment, the specific activity of the excreta calcium was lower in the vitamin D-treated chicks. It was concluded that one of the direct effects of vitamin D was the retention of calcium in actively growing bone.

Jones and Copp (1948), working on rats with Sr^{89} have shown a greater uptake of injected strontium by normal bone, although the difference in uptake between rachitic and normal bone does not occur until 30 min. after strontium dosage. Greenberg (1945) worked on rats with both Ca^{45} and Sr^{89} and concluded from his observations of treatment groups consisting of two rats that vitamin D exerted a direct effect on the mineralization of bone and only an indirect effect on the absorption of calcium.

This study investigates the uptake of oral and injected doses of Ca^{45} by nonrachitic and rachitic chicks. The observations do not confirm those noted by Greenberg (1945) on rats.

EXPERIMENTAL

One-day-old Leghorn chicks were divided into two large groups. One group received A. O. A. C. (1940) rachitogenic diet plus 50 units vitamin D/100 g. diet; the other group was fed the same diet plus corn oil. On the tenth day, in order to increase the severity of the rickets, all chicks were placed on a low calcium modification of the A. O. A. C. diet [Migicovsky and Emslie (1947)]. Vitamin D was increased to 100 units/100 g. diet and corn oil was continued as above.

At 15 days of age all chicks were dosed with Ca^{45} as CaCl_2 . One half the rachitic and nonrachitic chicks received an oral dose of 0.8 mg. Ca with an activity of 580,000 counts/min. The other half were injected intramuscularly with 0.4 mg. Ca with an activity of 290,000 counts/min.

The chicks were sampled at random at definite intervals after calcium dosage. Two samples per treatment per interval were taken, and five chicks represented a sample. The chicks were killed with chloroform, the left tibiae were excised and pooled in groups of five. The bones were ashed in a muffle furnace at 850°C ., the ash was dissolved in 4 N HCl and made up to volume.

The tibia ash was analyzed for Ca by permanganate titration of the oxalate.

The radioactivity measurements were made on the calcium oxalate precipitate. The precipitate was collected on a No. 50 Whatman filter-paper pad, which was then stuck to a celluloid disc with acetone in order to keep it flat. The preparation was then counted in a nucleometer. The amount of calcium on the pad was determined by pulling the pad away from the celluloid disc and titrating the oxalate with permanganate. Self-absorption corrections were applied to the readings.

DISCUSSION

The results of a representative experiment are shown in Tables I and II and a statistical analysis of the data is shown in Table III.

TABLE I

Deposition in the Tibia from an Oral Dose^a of 0.8 mg. Ca^{45} per Chick

| Non-rachitic | | | | Rachitic | | |
|-----------------------|-----------|-------------------|----------------------------|----------|-------------------|----------------------------|
| Interval after dosage | Ca, tibia | Specific activity | Activity retained in tibia | Ca tibia | Specific activity | Activity retained in tibia |
| days | mg. | counts/min./mg. | per cent | mg. | counts/min./mg. | per cent |
| 1 | 40.00 | 744 | 5.13 | 24.69 | 422 | 1.80 |
| 3 | 43.82 | 664 | 5.02 | 26.81 | 349 | 1.61 |
| 4 | 49.06 | 571 | 4.83 | 25.82 | 361 | 1.61 |
| 5 | 56.25 | 522 | 5.06 | 29.00 | 398 | 1.99 |
| 7 | 61.68 | 518 | 5.51 | 28.44 | 438 | 2.15 |
| 10 | 67.44 | 440 | 5.12 | 30.44 | 424 | 2.22 |

^a Equivalent to 580,000 counts/min.

It is conclusively illustrated that vitamin D exerts an effect on the oral calcium insofar as its deposition in bone is concerned. This difference between rachitic and nonrachitic tibiae is undoubtedly an absorption effect.

It is equally obvious that with the intramuscular dose no difference exists between the rachitic and nonrachitic chicks with respect to the total radioactivity per tibia. The specific activity (counts/min./mg. Ca) is greater in the rachitic bones, due to the larger size of the non-rachitic bone.

These results indicate that vitamin D does not exert a direct mineralization effect. If it did, we would expect to observe similar differences between rachitic and nonrachitic bones in the uptake of calcium from an injected dose as from an oral dose.

TABLE II

Deposition in the Tibia from an Intramuscular Dose^a of 0.4 mg. Ca⁴⁵ per Chick

| Non-rachitic | | | | Rachitic | | |
|-----------------------|-----------|-----------------------|----------------------------|-----------|-----------------------|----------------------------|
| Interval after dosage | Ca, tibia | Specific activity | Activity retained in tibia | Ca tibia | Specific activity | Activity retained in tibia |
| <i>days</i> | <i>mg</i> | <i>counts/min /mg</i> | <i>per cent</i> | <i>mg</i> | <i>counts/min /mg</i> | <i>per cent</i> |
| 1 | 39.25 | 406 | 5.50 | 23.62 | 689 | 5.61 |
| 3 | 43.62 | 389 | 5.85 | 26.32 | 668 | 6.06 |
| 4 | 45.44 | 385 | 6.03 | 26.00 | 643 | 5.76 |
| 5 | 51.62 | 344 | 6.12 | 25.62 | 678 | 5.99 |
| 7 | 61.25 | 298 | 6.29 | 28.81 | 668 | 6.64 |
| 10 | 73.38 | 257 | 6.50 | 27.19 | 672 | 6.30 |

^a Equivalent to 290,000 counts/min.

The decreasing specific activity values with time, of the nonrachitic chicks is due to the continuous deposition of nonradioactive dietary calcium. This phenomenon is demonstrated more clearly with an experiment conducted with toe bones, where the left toe serves as the control for the right toe.

Chicks were raised on low calcium A. O. A. C. diet with 100 units vitamin D/100 g. diet. An oral dose of Ca⁴⁵ was administered at 2 days of age and the terminal three phalanges of the third toe of the left foot (left toe) was biopsied 1 day after dosage. The similar bones of the right foot were then removed at a specified time after the left toe had been

TABLE III
Analysis of Variance of Per Cent Activity Retained in Tibia

| Source of variation | Degrees of freedom | Mean square | F |
|---|--------------------|-------------|------------------|
| Oral <i>vs.</i> intramuscular | 1 | 77.22 | 505 ^a |
| Rachitic <i>vs.</i> nonrachitic (oral) | 1 | 63.18 | 413 ^a |
| Rachitic <i>vs.</i> nonrachitic (intramuscular) | 1 | 0.01 | 0.06 |
| Interval: oral rachitic | 5 | 0.144 | 0.94 |
| Interval: oral nonrachitic | 5 | 0.066 | 0.43 |
| Interval: intramuscular rachitic | 5 | 0.310 | 2.03 |
| Interval: intramuscular nonrachitic | 5 | 0.284 | 1.86 |
| Error | 24 | 0.153 | |

^a Significant at $P = 0.01$.

removed. The terminal phalanx was discarded and the second and third retained for analysis. Thus we are able to observe the dilution of Ca^{45} or removal of Ca^{45} from the toe bone during the interval between left and right toe removal. The results of this experiment are shown in Table IV.

The data demonstrate quite clearly that the decrease in specific activity with time is largely due to a dilution of the bone calcium with non-radioactive dietary calcium. It is seen that only a small portion of the

TABLE IV
Dilution Effect of Deposited Dietary Calcium on the Specific Activity^a

| Interval between toe removal ^b | Ca/toe | | Specific activity ^c | | Total activity ^c | |
|---|------------|------------|--------------------------------|-------|-----------------------------|-------|
| | Left | Right | Left | Right | Left | Right |
| <i>days</i> | <i>mg.</i> | <i>mg.</i> | | | | |
| 1 | 1.31 | 1.38 | 900 | 889 | 1173 | 1214 |
| 3 | 1.24 | 1.34 | 1069 | 961 | 1323 | 1285 |
| 6 | 1.12 | 1.62 | 1142 | 806 | 1283 | 1304 |
| 9 | 1.14 | 2.11 | 1282 | 679 | 1469 | 1423 |
| 12 | 1.19 | 2.81 | 1103 | 412 | 1316 | 1159 |
| 15 | 1.25 | 3.47 | 1219 | 386 | 1525 | 1327 |

^a Each value represents the mean of four groups of five chicks per group.

^b All left toes were removed at 3 days of age.

^c Counts/minute/milligram.

previously deposited Ca^{45} is lost from the bone as shown by the difference in total radioactivity of left and right toes.

Experiments, with this technique, to study the factors affecting the movement of calcium into and out of bone are under way.

SUMMARY

Experiments on the deposition of calcium in rachitic and nonrachitic chick tibia from oral and intramuscular doses indicate that vitamin D does not exert a direct effect on the mineralization of bones of chicks.

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The Effects of Increased Potassium Concentration on the Metabolism of Rat Cerebral Cortical Slices^{1, 2}

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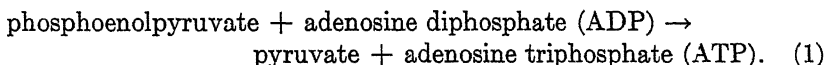
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INTRODUCTION

The intermediary metabolism of brain cortex slices is known to be sensitive to changes in the potassium-ion concentration of the medium. In 1935, Ashford and Dixon (1), working with rabbit brain cortex, discovered that the potassium salt of α - or β -glycerophosphate produced within certain concentration limits a marked increase in both the rate of aerobic glycolysis and the respiration, while the corresponding sodium salts had no such effect. This potassium effect was confirmed in the same year by Dickens and Greville (5), who reported a 50–100% increase in Q_{O_2} and $Q_G^{O_2}$ when 0.1 *M* KCl was added to slices of rat brain cortex respiring in glucose, fructose, lactate, or pyruvate. There was no increase in the absence of substrate. Both groups of investigators noted that the anaerobic glycolysis of brain slices was depressed by the potassium ion.

Potassium is now recognized to be capable of activating certain enzyme systems which are components of the glycolytic system. Recently, Boyer, Lardy, and Phillips (2, 3) have shown that with enzyme preparations from rat skeletal muscle, potassium in addition to Mg^{++} or Mn^{++} is required for the transphosphorylation



Lardy and Ziegler (8) were also able to demonstrate that reaction (1) is reversible. Meyerhof and Oesper (9) have confirmed the production

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² With the technical assistance of Philip B. Hollander.

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of phosphopyruvate from pyruvate by using C^{14} -labeled pyruvate and a purified transphosphorylase from rabbit muscle.

The situation with respect to alcoholic fermentation in yeast juice appears to be somewhat different. Muntz (10) concluded that in yeast the potassium-sensitive step occurs between glucose and ADP rather than between phosphoenolpyruvate and pyruvic acid.

This paper describes some metabolic effects of potassium on brain slices. The data are interpreted as suggesting that in brain, phosphoenolpyruvate rather than pyruvate is the link between the glycolytic chain and the tricarboxylic acid cycle.

EXPERIMENTAL

Methods and Materials

The brains of normal adult rats of the dark-hooded variety obtained from our stock colony were promptly removed after decapitation of the animal, chilled on ice, and placed on a cold cutting platform. Thin slices of cortical tissue were removed using a lucite template and razor blade. The tissue was weighed, and 10-mg. samples were introduced into chilled Warburg vessels of approximately 5 ml. capacity and containing 0.9 ml. medium. The whole procedure from the decapitation of the animal to the temperature equilibration of the vessels required between 15–20 min. Oxygen was used as the gas phase. All substrates were made up in a modified Krebs-Ringer phosphate solution (12) where the usual calcium concentration was lowered to $4.5 \times 10^{-4} M$, and the pH adjusted to 7.4. Potassium hydroxide (2 *N*) was used in the center well. The rate of oxygen uptake was measured at 10-min. intervals over periods of 30–60 min. Only linear data were accepted for calculations. Bath temperature was 37.5°C. unless otherwise noted.

Materials from the following sources were used: amino acids, Eastman Kodak Co.; α -ketoglutaric acid, Bios chemicals, recrystallized from acetone-benzene solution; glutamine, Delta Laboratories, NH_3 -free; pyruvic acid Eastman, recrystallized as the sodium salt after Robertson (11); ATP, Delta Laboratories. Other chemicals were standard brands, c.p. grade.

Potassium Effect on Glucose and Pyruvate Metabolism

Cerebral cortical slices were incubated with 0.011 *M* glucose for 30–40 min. until the control rate of oxygen uptake was established. Then a weighed amount of solid KCl in the side arm was mixed with the medium so that the final potassium concentration was 0.1 *M*. Another series of readings was taken for 40–50 min. to determine the new Q_{O_2} . As may be seen from Table I, the addition of 0.1 *M* KCl to brain slices in glucose produced an average increase in Q_{O_2} of 74% in

TABLE I

Effect of KCl on Q_{O_2}

Each substrate was added at 0.01 M concentration. Temperature 37.5°C.

| Substrate | No expts. | Av Q_{O_2} , control period | Subst added | Av Q_{O_2} , exptl period | Average change |
|--|--------------|-------------------------------------|---------------|-----------------------------------|-------------------|
| Glucose | 12 | 10.04 | 0.1 M KCl | 17.46 | $\%_c$ +74 |
| | 2 | 12.16 | 0.1 M Sucrose | 12.84 | +5 |
| L-Glutamate | 2 | 5.99 | 0.1 M KCl | 4.69 | -22 |
| | | | 0 | 4.60 | -23 |
| Glucose + L-glutamate | 6 | 12.01 | 0.1 M KCl | 12.19 | +1 |
| Glucose + citrate | 2 | 18.54 | 0.1 M KCl | 19.37 | +4 |
| α -Ketoglutarate | 2 | 4.46 | 0.1 M KCl | 4.03 | -10 |
| Glucose + α -ketoglutarate | 2 | 14.22 | 0.1 M KCl | 15.20 | +6 |
| Succinate | 6 | 9.16 | 0.1 M KCl | 5.89 | -36 |
| | | | 0 | 6.20 | -33 |
| Glucose + succinate | 5 | 16.97 | 0.1 M KCl | 16.93 | -1 |
| Glucose + succinate + .025 M malonate | 2 | 10.78 | 0.1 M KCl | 14.80 | +37 |
| Glucose + L-aspartate | 4 | 10.26 | 0.1 M KCl | 15.68 | +53 |
| Glucose + DL-methionine | 2 | 9.33 | 0.1 M KCl | 20.33 | +117 |
| Glucose + L-glutamine | 4 | 14.50 | 0.1 M KCl | 21.12 | +45 |

twelve experiments. Conceivably, this addition of KCl might have caused an increase in respiration through osmotic effects. However, repetition of the procedure using sucrose instead of KCl produced no significant change in respiratory rate. Lactate and pyruvate showed definite but somewhat less marked responses to potassium (Table II), while with α -ketoglutarate or succinate as substrate, no such increase was noted (Table I).

Inhibition of the Potassium Effect

Krebs and Eggleston (7) have recently reported that active transport of the potassium ion into guinea pig brain cells occurs only when both glucose and glutamate are available to the cells. Therefore it seemed of value to determine whether the addition of glutamate to the glucose substrate would influence the potassium effect. It was found that in a medium containing both glucose and glutamate, the Q_{O_2} response to added KCl was abolished. There was likewise no stimulating effect of KCl on the Q_{O_2} in glutamate alone (Table I).

Brain slices in lactate and pyruvate media gave a lower Q_{O_2} , but a greater percentage potassium response at 25.5°C. than at 37.5°C. (Table II). Moreover, while the addition of glutamate caused no inhibition of the potassium effect at 37.5°C., there was evidence of some inhibition at the lower temperature. Comments on this experiment appear in a later section.

A number of carbohydrate intermediates were also tested on the glucose-KCl system (Table I). It was found that the addition of citrate, α -ketoglutarate, or succinate would prevent the Q_{O_2} increase with potassium. In the case of succinate, the addition of malonate (0.025 *M*) caused a reappearance of the response to potassium.

TABLE II

Effect of Temperature on Q_{O_2} Response to KCl in Lactate and Pyruvate Substrates
From these data, the Q_{10} for pyruvate substrate is 3.0,
that for L-glutamate substrate is 1.20.

| Substrate | No. expts. | Av. Q_{O_2} , control period | Subst. added | Av. Q_{O_2} , exptl. period | Average change |
|------------------------|------------|--------------------------------|------------------|-------------------------------|----------------|
| Temp. 37.5°C. | | | | | |
| Lactate | 4 | 11.53 | 0.1 <i>M</i> KCl | 13.61 | +18 |
| Lactate + L-glutamate | 4 | 15.47 | 0.1 <i>M</i> KCl | 17.49 | +13 |
| Pyruvate | 4 | 15.54 | 0.1 <i>M</i> KCl | 19.75 | +27 |
| Pyruvate + L-glutamate | 4 | 17.72 | 0.1 <i>M</i> KCl | 24.11 | +35 |
| L-Glutamate | 2 | 5.99 | 0.1 <i>M</i> KCl | 4.69 | -22 |
| | | | 0 | 4.60 | -23 |
| Temp. 25.5°C. | | | | | |
| Lactate | 6 | 3.51 | 0.1 <i>M</i> KCl | 7.68 | +118 |
| Lactate + L-glutamate | 6 | 5.91 | 0.1 <i>M</i> KCl | 7.39 | +25 |
| Pyruvate | 4 | 4.33 | 0.1 <i>M</i> KCl | 8.39 | +93 |
| Pyruvate + L-glutamate | 4 | 4.87 | 0.1 <i>M</i> KCl | 7.71 | +58 |
| L-Glutamate (27.5°C.) | 3 | | 0 | 3.84 | |

To discover whether the inhibitory effect of glutamate might be due to its ability to enter a tricarboxylic acid cycle through conversion to α -ketoglutarate, other related compounds which were not reactants of the cycle were also tested. Aspartic acid, methionine, or glutamine had no influence on the course of the KCl response in glucose.

In general, then, it seems that the increase in oxygen uptake on addi-

TABLE III

Effect of Inhibitors on Q_{O_2} Response to Added KCl
 All substrates present at a concentration of 0.01 M.
 Potassium chloride added to 0.1 M

| Inhibitor | Substrate | No. expts. | Q_{O_2} control | Q_{O_2} control, KCl | Change | Q_{O_2} with inhibitor | Q_{O_2} with inhibitor, KCl | Change |
|--|-----------------------|------------|-------------------|------------------------|---------|--------------------------|-------------------------------|---------|
| NaF 2×10^{-3} M | Glucose | 2 | 10.45 | 13.89 | % 32 | 8.39 | 8.27 | % -2 |
| | Lactate | 2 | 9.10 | 12.47 | 37 | 9.17 | 8.92 | -2 |
| DNP 8×10^{-5} M | Glucose | 4 | 14.32 | 20.20 | 41 | 15.83 | 16.41 | +3 |
| | Pyruvate | 2 | 16.68 | 20.26 | 21 | 17.26 | 16.25 | -6 |
| NaN ₃ 6×10^{-3} M | Glucose | 2 | 9.80 | 15.62 | 59 | 3.45 | 5.52 | +60 |
| | Glucose + L-glutamate | 2 | 14.00 | 13.55 | -4 | 3.74 | 4.75 | +27 |

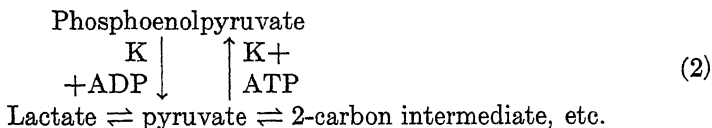
tion of KCl to rat brain slices respiring in glucose is abolished by the presence of members of the tricarboxylic acid cycle or by substances giving rise to appreciable amounts of such intermediates.

Among the enzyme inhibitors tried, sodium fluoride (2×10^{-3} M) and 2,4-dinitrophenol (8×10^{-5} M) prevented the rise in Q_{O_2} caused by KCl. Sodium azide (6×10^{-3} M) had no such effect (Table III).

DISCUSSION

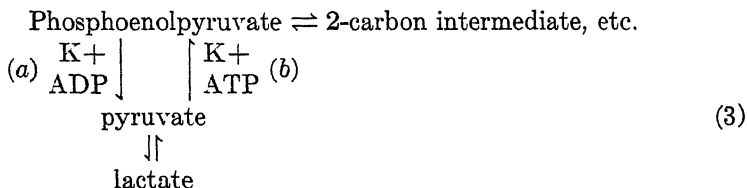
An interpretation of these experiments is handicapped by the present lack of information concerning the intermediary metabolism of brain. There is some evidence to indicate the normal occurrence in brain of glycolytic mechanisms leading to the production of both lactate and pyruvate under aerobic conditions in the intact animal (6). It is not known definitely, however, whether in resting brain the aerobic metabolism of pyruvate itself normally proceeds beyond this point, or if so, whether it proceeds via the tricarboxylic acid cycle. Recently Coxon, Liébecq, and Peters (4) have reported an increased accumulation of citrate in a dialyzed pigeon brain homogenate with pyruvate as substrate and in the presence of fumarate. They interpreted their results to indicate the entrance of pyruvate into some form of the tricarboxylic acid cycle.

Assuming the existence of such a cycle in brain, the results of the experiments here reported may be interpreted on the basis of the known potassium catalysis of the phosphopyruvate-ADP transphosphorylation as worked out by Boyer, Lardy, and Phillips (2,3) and by Lardy and Ziegler (8). If the usual glycolytic scheme is invoked, there is no reason to expect potassium to have any effect on the pyruvate Q_0 , since the potassium-sensitive step is not involved, *i.e.*:



The same argument would apply with lactate as the substrate.

If we postulate, however, that pyruvate, like lactate, is found *in quantity* only during anaerobic glycolysis or in conditions such as thiamine deficiency, and is not an abundant substance in a normal and aerobic metabolic environment, then we may postulate the following scheme, which would explain the effect of added potassium in either a lactate or a pyruvate medium:



This concept would make phosphopyruvate rather than pyruvate the pivotal point between the glycolytic steps and the tricarboxylic acid cycle.

This postulate is useful in explaining a number of the experimental results reported here. It explains the increased Q_0 , with lactate or pyruvate as substrate in the presence of added potassium. It may also serve to explain the potassium effect when glucose is the substrate. Under these circumstances, stimulation of reaction (1) by the added potassium would result in an increased rate of ATP generation, thus leading to a more rapid turnover of glucose. Of course, it would also be possible to explain the increased glucose utilization with added potassium by assuming, as Muntz does (10) for yeast, the existence of a

potassium-sensitive reaction in the glycolytic system at a level above the phosphopyruvate state. This assumption would not explain the results with pyruvate. Our experiments do not exclude the possibility of an additional potassium-sensitive reaction at the higher level, but the postulation of this second site seems unnecessary.

The concept of the phosphopyruvate link also permits some explanation of the inhibition of the potassium effect by glutamate and the intermediates of the tricarboxylic acid cycle which were tried. These substances yield large amounts of high-energy phosphate during their metabolic degradation. With this increased ATP level available to initiate the phosphorylation of glucose, the additional supply of ATP resulting from the potassium effect on the phosphopyruvate reaction would have little influence on the rate of glucose phosphorylation, as measured here by the Q_{O_2} .

On the other hand, the potassium effect on pyruvate and lactate would not be inhibited by these substances even if the level of ATP were a limiting factor, since the additional supply available from the oxidation of these intermediates should increase the rate of the potassium-sensitive step in the direction of phosphopyruvate, and thus promote a higher Q_{O_2} , if anything. At a lower temperature, however, where the rate of respiration is diminished, a change in relative enzyme activities as a result of differing temperature coefficients might cause glutamate to become the chief energy source in the cell even though pyruvate is still present. The temperature coefficients for the utilization of pyruvate and of glutamate were determined, and it was found (Table II) that while the Q_{10} for pyruvate was approximately 3, that for glutamate was 1.2. Therefore in pyruvate-glutamate mixtures at low temperatures, glutamate is contributing a larger proportion of the substrate oxidized, resulting in a respiration less sensitive to increased potassium concentration. This explains the apparent inhibition of the potassium effect which was found at the lower temperature.

If the proposed scheme is correct, there should be some ratio of glucose and pyruvate substrates at which steps (a) and (b) in reaction 3 should be exactly balanced, so that an increment in potassium concentration will have no effect on the relative amounts of pyruvate and phosphopyruvate, nor on the Q_{O_2} in the mixed substrate. This experiment was done, and the results may be found in Fig. 1. It may be seen that the presence of $2 \times 10^{-4} M$ pyruvate can cut the potassium effect on $0.011 M$ glucose down to 10% of its usual value, although the absolute

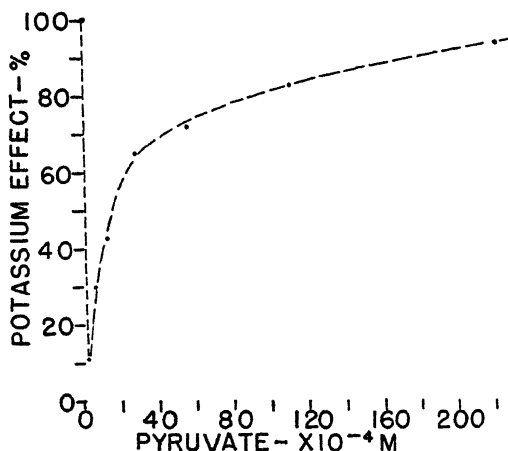


FIG. 1. Effect of pyruvate on Q_{O_2} of rat brain slices in glucose-KCl medium. Medium contains 0.01 *M* glucose in Krebs-Ringer phosphate, with sodium pyruvate added as noted. The increase in Q_{O_2} resulting from the addition of 0.1 *M* KCl to slices in glucose medium is taken as 100% potassium effect, and the other values are calculated on this basis. Temperature 37.5°C.

value of the resting Q_{O_2} is only slightly raised by this concentration of pyruvate. Thus it appears that there is an antagonism between glucose and pyruvate which can be adequately explained by assuming that phosphopyruvate is the pivotal point between anaerobic and aerobic systems.

ACKNOWLEDGMENT

We wish to thank Prof. Richard J. Winzler of the University of Southern California for his suggestions and criticisms in the preparation of this paper.

SUMMARY

1. The marked stimulation (50–100%) of oxygen uptake produced by the addition of 0.1 *M* KCl to rat brain cortical slices respiring aerobically in glucose, pyruvate, or lactate medium is confirmed. Potassium chloride has no effect when succinate, L-glutamate, or α -ketoglutarate is the substrate.

2. Using glucose substrate, this stimulation is shown to be inhibited by the addition of 0.01 *M* citrate, α -ketoglutarate, L-glutamate, or succinate. The effect of the latter is reversed by malonate. No inhibition was observed with L-aspartate, L-glutamine, or DL-methionine. The po-

tassium effect can be inhibited with sodium fluoride or 2,4-dinitrophenol.

3. Using pyruvate or lactate as substrate, the KCl stimulation of the Q_{O_2} is shown to be unaffected by any of the substances mentioned above in item 2 except fluoride or dinitrophenol, at a reaction temperature of 37.5°C. At 25.5°C., however, L-glutamate produces approximately a 50% reversal of the potassium effect.

4. At a given glucose concentration, the magnitude of the potassium effect varies with the pyruvate concentration, reaching a minimum at about 2×10^{-4} M pyruvate.

5. These results are discussed in terms of the potassium-requiring phosphopyruvate-adenosine diphosphate transphosphorylation. The possibility of phosphopyruvate being the linking intermediate between the glycolytic steps and the tricarboxylic acid cycle is suggested.

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Studies on the Mechanism of Protein Synthesis in Embryonic and Tumor Tissues. II. Inactivation of Fetal Rat Liver Homogenates by Dialysis, and Reactivation by the Adenylic Acid System ¹

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INTRODUCTION

One advantage which homogenates possess over tissue slices for the study of the mechanism of amino acid incorporation into protein is that it is easier to remove essential soluble metabolites from the former system. Homogenates which have been inactivated in this manner may subsequently be reactivated by adding appropriate known substances.

Bloch (1) found that the synthesis of glutathione from its constituent amino acids in pigeon liver homogenates could be markedly stimulated by adding adenosine triphosphate (ATP), succinate, or fumarate. However, the first attempts of the author to stimulate the incorporation of amino acids into the protein of rat liver homogenates by adding various metabolites (including ATP) were unsuccessful, owing apparently to the fact that such substances were already present in adequate concentration. Likewise Borsook and associates (2) noted that neither high-energy phosphates (ATP and 2-phosphoenol pyruvate), nor Krebs citric acid intermediates, affected the rate of lysine-6-C¹⁴ incorporation into protein of guinea pig liver homogenate. A twofold stimulation of glycine-1-C¹⁴ uptake by rat liver homogenate was observed upon adding a mixture of L-amino acids (3), but this effect was not obtained with fetal liver, owing probably to the relatively high amino acid concentrations prevailing in embryonic tissues.

¹ Aided by a grant from the Iowa Division of the American Cancer Society.

Indirect evidence that amino acid incorporation into proteins is associated with phosphorylation reactions was advanced by Frantz and co-workers (4) on the basis of their finding that addition of dinitrophenol inhibited the uptake of labeled alanine into liver slice protein, while permitting oxygen consumption to occur.

Recently Peterson, Winnick, and Greenberg reported (5) that a fraction of cytoplasmic granules, obtained by differential centrifugation of rat liver homogenate, had a low activity when drained free of the liquid phase. But upon adding a medium containing ATP, magnesium ions, citrate, and a mixture of (nonlabeled) L-amino acids, the activity of the granules with respect to the incorporation of single labeled amino acids into protein was increased as much as sevenfold. This same degree of activity was obtained by adding to the granules some of the particle-free supernatant liquid, derived from high-speed centrifugation of a concentrated homogenate.

In seeking to extend the evidence that certain metabolites, including the adenylic acid system, are essential for the amino acid incorporation process, the author has chosen a somewhat different system, fetal liver, because of its relatively high activity, and has employed a different means of removing essential metabolites, namely dialysis. Fetal rat liver homogenates, inactivated by dialysis against isotonic salt solutions, regain most of their ability to incorporate C^{14} -labeled amino acids when supplied with adenosine phosphates under aerobic conditions. The degree of reactivation is increased by adding also an amino acid mixture and certain intermediates of the Krebs citric acid cycle.

EXPERIMENTAL

Labeled Amino Acids

Glycine-1- C^{14} and DL-alanine-1- C^{14} were obtained by allocation from the U. S. Atomic Energy Commission, and purchased from Tracerlab, Inc., Boston. Solutions of these compounds were prepared, and their radioactivity per milligram carbon measured, as described in the preceding paper of this series (6).

Preparation of Homogenates

The techniques were those already given (6), except that the fetal livers were homogenized with three parts of a modified Krebs-bicarbonate solution. This solution was prepared in the usual manner (7), except that NaCl and $NaHCO_3$ were replaced by equivalent amounts of KCl and $KHCO_3$. The solution was saturated with 95% O_2 :5% CO_2 gas.

Two rats, pregnant for 17–18 days, were employed for a series of assays, and usually yielded a total of 2–3 g. of fetal livers, sufficient for 20–25 incorporation experiments.

Dialysis Technique

A 1-ml. portion of the homogenate was aged at 0° as a nondialyzed control. The remainder was transferred to a thin (cylindrical) cellophane tube, 1.8 cm. in diameter. The latter was attached to a glass rod, which extended into the tube. The upper end of the rod was fastened to an electric stirrer. The cellophane tube (with stirrer attached) was lowered into a glass cylinder containing 400 ml. of modified Krebs solution. The latter, in turn, was surrounded by an ice bath. The cellophane tube was then rotated at about 200 r.p.m. for a standard time interval of 2.5 hr., unless otherwise stated.

Incubation Procedure

Specified metabolites, in solutions of pH 7.5, which were stored frozen when not in use, were added to 12-ml. centrifuge tubes in quantities needed to give the final concentrations desired in the homogenate systems. Also, 0.15 mg. glycine-1-C¹⁴ or 0.36 mg. DL-alanine-1-C¹⁴ was added, to provide 0.005 *M* or 0.01 *M* concentrations, respectively (following the addition of 0.4 ml. of homogenate). The tubes were then dried *in vacuo*. To each tube thus prepared was added 0.4-ml. portions of aged or dialyzed homogenate (equivalent to 0.1 g. liver), and the same incubation technique previously described (6) was used. A standard incubation time of 2 hr. (under 95% O₂:5% CO₂) was employed in all cases. About 10 mg. of dry protein powder was isolated from each tube and its specific radioactivity determined, as described in (6). The radioactivity was expressed in $\mu\text{g. of C}^{14}$ incorporated/g. protein/2 hr.

RESULTS

Freshly prepared fetal liver homogenates from different pregnant rats generally incorporated 20–30 $\mu\text{g. C}^{14}$ /g. protein/2 hr., when incubated with glycine-1-C¹⁴, and 18–25 $\mu\text{g. C}^{14}$ with alanine-1-C¹⁴. Aging the homogenates at 0° for 3 hr. (without dialysis) reduced their activity by only about 5–10%.

Figure 1 shows that the homogenates rapidly lost their ability to incorporate labeled glycine or alanine upon dialysis at 0°. After 3 hr. the inactivation amounted to about 80–90%. In preliminary experiments with a more concentrated (1:1) homogenate, of the type used in previous work, the dialysis was much slower, due probably to inadequate internal agitation.

In studying the reactivation of dialyzed homogenates, a standard dialysis time of 2.5 hr. was arbitrarily employed. This time interval generally corresponded to a loss of 80% of the incorporative activity, as compared with an undialyzed control, aged at 0° (Fig. 1). If longer dialyses were employed, reactivation became more difficult.

Fig. 2, curve 1 shows that the addition of ATP restored a substantial portion of the activity with respect to glycine-1- C^{14} uptake. The maximum response, at approximately 0.0015 M ATP concentration, represents about a threefold increase above the basal level. Curve 2 shows that an additional stimulation results when 0.4 mg. of a mixture of L-amino acids (Table I)² is added, together with ATP and Mg^{++} . With this combination, incorporative activity of the homogenate reached 80% of that for the undialyzed control. Adenosine triphosphate exerted a marked inhibition at higher concentrations (of the order of 0.005 M).

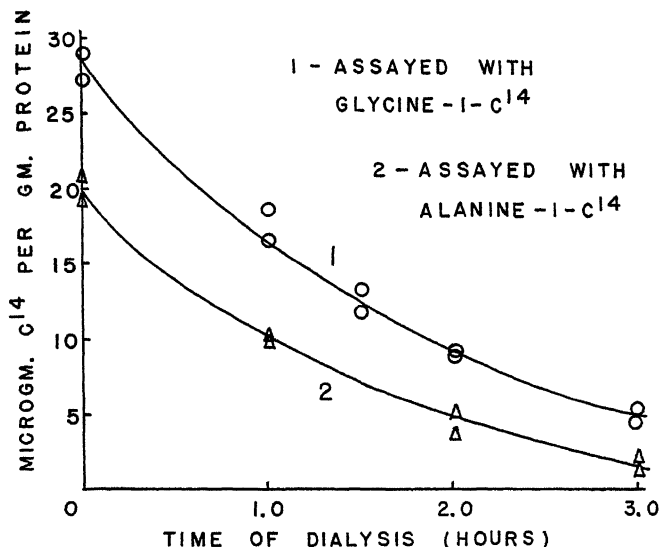


FIG. 1. Rate of loss of incorporative activity upon dialysis of homogenates. Aliquots were removed at various times (0.4 ml.) and assayed by incubation with labeled amino acid.

In Fig. 3, curve 1 shows that the dialyzed homogenates were only slightly stimulated by the L-amino acid mixture, in the absence of ATP. The combination of ATP and amino acid mixture (curve 2) again restored about 80% of the incorporative activity.

² The composition of this mixture is arbitrary, and intended to represent the approximate proportions of the amino acids in typical proteins. The importance of each of the individual components has not yet been determined.

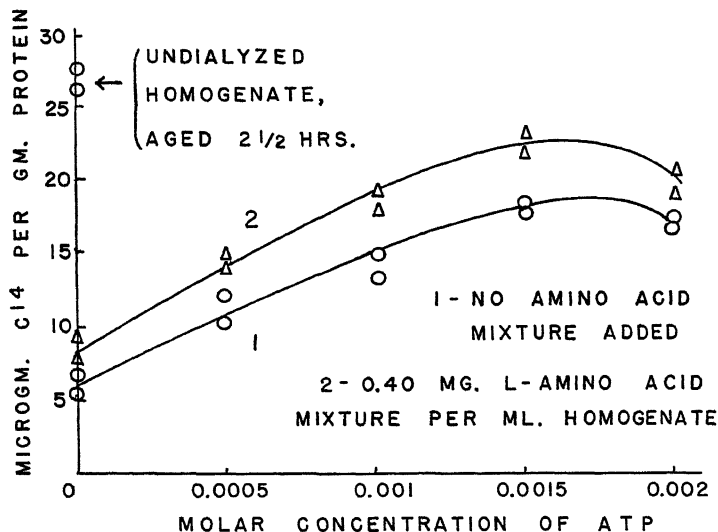


Fig. 2. Stimulation by ATP of glycine-1-C¹⁴ incorporation in dialyzed homogenates. Mg⁺⁺ (0.006 M) was added to each tube.

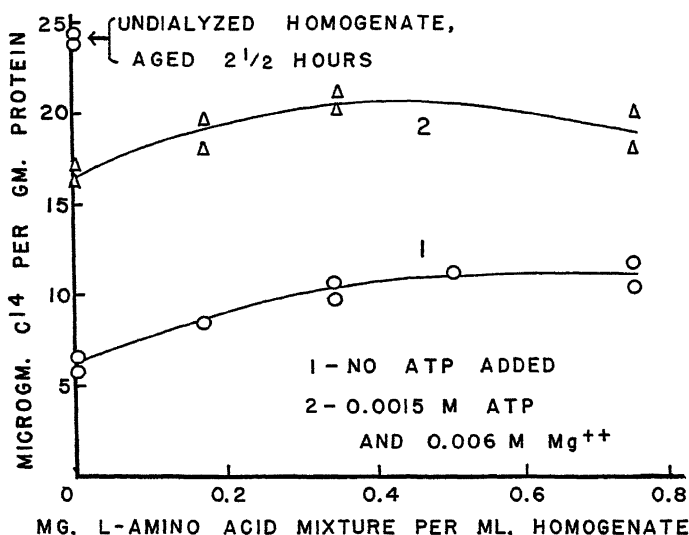


Fig. 3. Effect of L-amino acid mixture on glycine-1-C¹⁴ uptake in dialyzed homogenates.

The stimulatory effect of several phosphorylated compounds on the incorporation of glycine-1-C¹⁴ and alanine-1-C¹⁴ is shown in Table II. Adenosine diphosphate (ADP) and adenosine-5-phosphate (AMP) are equally as effective as ATP. L-Glycerophosphoric acid, which appears to function as an ATP generator, is slightly less effective under the conditions employed. As would be expected, adenosine exerted no significant effect.

TABLE I

Composition of L-Amino Acid Mixture

The mixture was dissolved with the addition of dilute KOH to give a pH of 7.5. Glycine and serine were omitted from the mixture when the latter was intended for use with glycine-1-C¹⁴. Similarly alanine was omitted when the mixture was used with alanine-1-C¹⁴.

| Amino acid | Solution mg./ml. |
|-----------------|---------------------|
| Glycine | 0.8 |
| L-Alanine | 0.8 |
| L-Serine | 0.8 |
| L-Threonine | 0.8 |
| L-Valine | 1.0 |
| L-Leucine | 1.2 |
| L-Isoleucine | 1.1 |
| L-Phenylalanine | 1.1 |
| L-Tyrosine | 1.0 |
| L-Proline | 0.6 |
| L-Tryptophan | 0.4 |
| L-Methionine | 1.0 |
| L-Cysteine-HCl | 1.0 |
| L-Glutamic acid | 1.8 |
| L-Aspartic acid | 1.2 |
| L-Arginine-HCl | 1.2 |
| L-Lysine | 1.0 |
| L-Histidine | 1.0 |

In connection with his demonstration that peptide bond formation was associated with energy-yielding reactions, Bloch (1) found that 0.01 *M* succinate and fumarate markedly stimulated glutathione synthesis in pigeon liver homogenates, while 0.01 *M* malonate was inhibitory. In the present study, the various intermediates of the Krebs citric acid cycle were first tested on freshly prepared, undialyzed homogenates and found to give either erratic stimulations, or to exert no effect on glycine-1-C¹⁴ uptake into protein. By using dialyzed homogenates to which

TABLE II

Stimulatory Effect of Phosphorylated Compounds on Amino Acid Incorporation in Dialyzed Homogenates

The adenosine, adenosine phosphates, and glycerophosphoric acid were employed at 0.0015 *M* concentration, Mg^{++} at 0.006 *M*, and the L-amino acid mixture at 0.4 mg./ml. homogenate, in all assays except the controls. The recorded values are averages of triplicate incubations.

| Substance added to dialyzed homogenate | C^{14} uptake in $\mu g./g.$ protein | |
|--|--|--------------------------|
| | With glycine-1- C^{14} | With alanine-1- C^{14} |
| Control: no additions | 4.7 | 3.9 |
| Adenosine | 5.8 | 4.2 |
| Adenosine-5-phosphate | 19.0 | 14.6 |
| Adenosine diphosphate | 19.6 | 15.0 |
| Adenosine triphosphate | 19.2 | 15.9 |
| L-Glycerophosphate | 15.9 | 12.9 |
| Control: undialyzed, aged at 0°, no additions | 22.6 | 20.0 |

TABLE III

Effect of Krebs Intermediates on Reactivation of Dialyzed Homogenates

Fetal rat liver was dialyzed and then assayed with glycine-1- C^{14} , as described in the experimental section. Adenosine triphosphate (ATP), 0.0015 *M*, 0.006 *M* Mg^{++} , and 0.4 mg. L-amino acid mixture/ml. homogenate, were employed in all assays, except the controls. Columns 2 and 3 represent separately performed experiments. The recorded values are averages of duplicate incubations. They are expressed in terms of the activity of the undialyzed homogenates.

| Krebs intermediate added to dialyzed homogenate | C^{14} uptake in $\mu g./g.$ protein | |
|---|---|--|
| | With intermediate at 0.005 <i>M</i> concentration | With intermediate at 0.01 <i>M</i> concentration |
| Control: undialyzed homogenate, aged at 0°, no additions | 100 ^a | 100 ^b |
| Control: no additions | 16.5 | 18.5 |
| None (ATP, Mg^{++} and amino acid mixture present) | 81 | 77 |
| Citrate | 87 | 86 |
| Ketoglutarate | 79 | 78 |
| Succinate | 92 | 89 |
| Fumarate | 83 | 84 |
| Malate | 86 | 93 |
| Oxalacetate | 81 | 80 |
| Pyruvate | 80 | 79 |

^a Corresponds to 28.8 $\mu g.$ $C^{14}/g.$ protein.

^b Corresponds to 24.7 $\mu g.$ $C^{14}/g.$ protein.

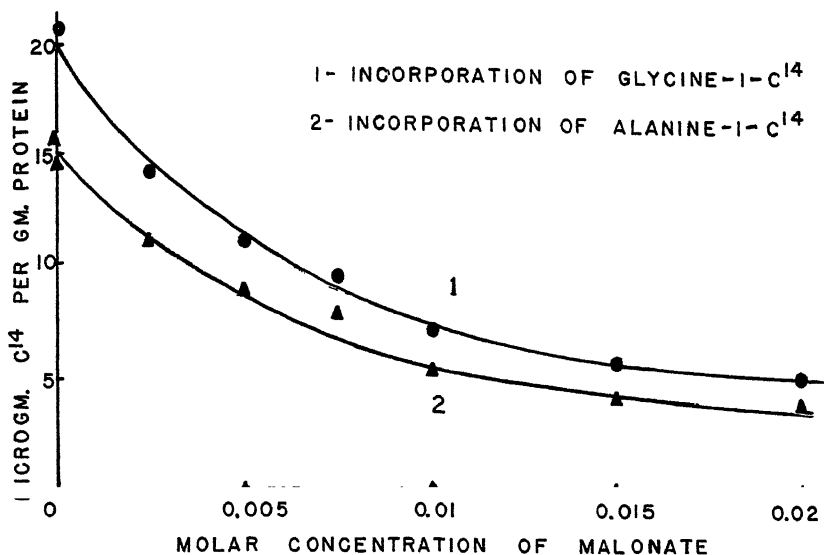


Fig. 4. Inhibition by malonic acid of amino acid incorporation in (undialyzed) homogenates.

ATP, Mg^{++} , and amino acid mixture were added (Table III), slight additional stimulations were consistently obtained with citrate, succinate, fumarate, and malate, at 0.005 M and 0.01 M concentrations. Ketoglutarate, oxalacetate, and pyruvate had no significant effect. In no case was the stimulation very pronounced. This is perhaps to be expected, since certain of the amino acids in the mixture added to the homogenates would be effective as Krebs intermediates for energy-yielding reactions. Most likely a sparing of ATP is involved here.

At higher concentrations (above 0.02 M), the individual Krebs intermediates partially inhibited incorporation of labeled glycine. It may be mentioned that a combination of the seven organic acids of Table III (0.01 M concentration), mixed in equal proportions, failed to stimulate glycine-1-C¹⁴ uptake significantly in dialyzed homogenates containing added ATP, Mg^{++} , and L-amino acid mixture. The inability to obtain 100% reactivation may be due to loss of such factors as coenzymes.

The inhibitory effect of malonic acid on glycine-1-C¹⁴ and alanine-1-C¹⁴ uptake is shown in Fig. 4. Most, though not all, of the incorporative activity of freshly prepared homogenates was abolished by 0.01–0.02 M malonate.

DISCUSSION

In a recent review, Borsook (8) emphasizes the great complexity of the problem of protein synthesis. The present experiments give no information as to the exact mechanism whereby high-energy phosphates promote amino acid incorporation. The simplest assumption is that the amino acids are themselves phosphorylated prior to combination in peptide linkages (9). It is also possible that the effect of high-energy phosphates may be indirect. Brenner, Muller, and Pfister (10) found that chymotrypsin could readily convert various aliphatic and aromatic esters of methionine into methionine peptides, and they suggest the hypothesis that the biological synthesis of peptide structures may involve the interaction of esterified amino acids. The energy for the synthesis of these esters could be supplied by way of substances like ATP.

While the exact nature of the amino acid incorporation process in slices and homogenates is unknown, there is little doubt that peptide bond synthesis occurs (6,8,11). As pointed out by Zamecnik and Frantz (11) and Borsook (8), most investigators at present avoid such terms as "turnover," "exchange," and "protein synthesis," in short-term experiments. However, the report by Peters and Anfinsen (12) of a net production of serum albumin in liver slices previously depleted of soluble proteins is significant. The possibility cannot be excluded that new protein molecules may be synthesized in slices and even in homogenates, particularly those derived from rapidly growing tissues. A major problem in this connection, posed most recently by Borsook (8), is whether proteins are built up with peptides as intermediates, or by a template mechanism.

SUMMARY

Homogenates of embryonic rat liver lost most of their ability to incorporate C^{14} -labeled glycine or alanine into protein upon being dialyzed against a modified Krebs-bicarbonate solution. Most of the incorporative activity could be subsequently restored by adding to the dialyzed homogenates adenosine triphosphate, magnesium ions, and a mixture of nonlabeled L-amino acids. Adenosine diphosphate and monophosphate were equally as effective as the triphosphate. Glycerophosphoric acid was slightly less effective. Adenosine had no appreciable effect. Certain of the Krebs citric acid intermediates exerted a slight additional stimulation when added together with the adenylic acid system and the amino acid mixture. Malonate was inhibitory.

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Pyrimidine Riboside Metabolism

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INTRODUCTION

Studies of the metabolism of pyrimidine ribosides have been limited compared with those on purine nucleosides (1,2). Information on the specificity of pyrimidine nucleosidase and the degradation of ribose from pyrimidine nucleosides has been lacking. Only recently has the specificity of cytosine nucleoside deaminase been studied (3). The following is a report on our attempts to explore this field.

Bacteria were used as the source of enzymes, since their high rate of nucleic acid metabolism makes them particularly suitable for such studies. A new technique for the determination of pyrimidine-bound ribose facilitated our work. It was found that pyrimidine riboside metabolism has much in common with that of purine ribosides. In particular, an identical role of phosphate in the splitting by nucleosidase was observed. The isolation of bacterial pyrimidine ribosidase in the cell-free state has made possible a study of this enzyme. Like purine ribosidase it shows high specificity.

Methods and Materials

(a) Substances

Cytidine was prepared by hydrolysis of commercial ribonucleic acid according to Bredereck (4). The product was recrystallized as the sulfate (N 14.7%, calcd. 14.35%; $E_m(270 \text{ m}\mu) = 8.7 \times 10^3$). Orotic acid was synthesized (5,6) (N 15.9%, calcd. 16.1%; $E_m(277 \text{ m}\mu) = 7.3 \times 10^3$). Cytosine was prepared by formic acid hydrolysis (7) of cytidine and isolated via the phosphotungstate [N 33.7%, calcd. 32.3%; $E_m(262 \text{ m}\mu) = 6.6 \times 10^3$]. Uracil, uridine, uridylic acid, and cytidylic acid were commercial products. Their identity was verified by spectrophotometric measurements.

(b) *Enzymes*

Cells of *Escherichia coli* served as a source of enzymes. Identical results were obtained with *Aerobacter aerogenes* and *Micrococcus lysodeikticus*. The culture medium for *E. coli* was composed as follows: peptonized milk, 15.0 g.; K_2HPO_4 , 7.5 g.; tap water, 100 ml.; distilled water, 900 ml.

Fresh Cells. The organisms were harvested after 20 hr. using a Sharples centrifuge, and washed two times with water.

Lyophilized Cells. Fresh cells were dehydrated in the frozen state and stored in a desiccator.

Acetone-Treated Cells. The paste of fresh cells was shaken thoroughly with 7 parts of ice cold acetone for 5 min. The cells were filtered off, suspended in 14 parts of acetone and shaken for 10 min. The second acetone treatment was repeated. After filtration, traces of adherent acetone were removed by evacuation.

Cell-Free Extract. The paste of fresh cells was mixed with glass powder and ground in the apparatus of Kalnitsky, Utter, and Werkman (8). The material was suspended in 0.05 *M* phosphate buffer, pH 7.0, and cleared from cell debris by centrifugation. An alternative was sonic vibration of acetone-treated cells with a Raytheon 9-kc. Magnetostriction Oscillator. One-half g. of cells was suspended in 20 ml. of 0.1 *M* phosphate buffer, pH 7.0, and exposed for 20 min. Centrifugation for 20 min. at 10,000 r.p.m. removed a small amount of debris. By precipitation with ammonium sulfate and dialysis further purification was accomplished.

(c) *Analytical Procedures*

Spectrophotometry. For spectrophotometric measurements, a Beckman instrument, model DU, was used. In the incubations with fresh cells, centrifugation was found sufficient to remove interfering material; considerable dilution prior to the measurements further reduced interference of inert material. With other enzyme preparations deproteinization with perchloric acid (9,10) was necessary.

TABLE I

Orcinol Pentose Test with Pyrimidine Nucleosides and Nucleotides

Experimental conditions as given by Meijbaum (13) and outlined in the text

| Substance examined <i>μg.-moles</i> | <i>E</i> (660m μ) observed |
|--|---------------------------------|
| Ribose 0.023 | 0.105 |
| Ribose 0.045 | 0.220 |
| Ribose 0.057 | 0.278 |
| Ribose 0.086 | 0.440 |
| Cytidine 0.248 | 0.013 |
| Cytidine 1.013 | 0.140 |
| Uridine 0.266 | 0.101 |
| Uridine 0.665 | 0.643 |
| Cytidylic acid 0.233 | 0.006 |
| Cytidylic acid 1.032 | 0.058 |
| Uridylic acid 0.204 | 0.041 |
| Uridylic acid 1.018 | 0.423 |

Phosphate Determination. Aside from the conventional Fiske and SubbaRow technique for determination of inorganic and organic phosphate, the Lowry and Lopez method for inorganic phosphate in the presence of labile organic phosphate was used (11). To avoid losses of labile organic phosphate in the perchloric acid deproteinization, the mixtures were partially neutralized by adding sodium acetate solution as specified by Lowry and Lopez (11), but prior to centrifugation. By this modification the samples were exposed to perchloric acid not longer than 1 min. The removal of protein may be less complete, but no interference with the phosphate determination was observed.

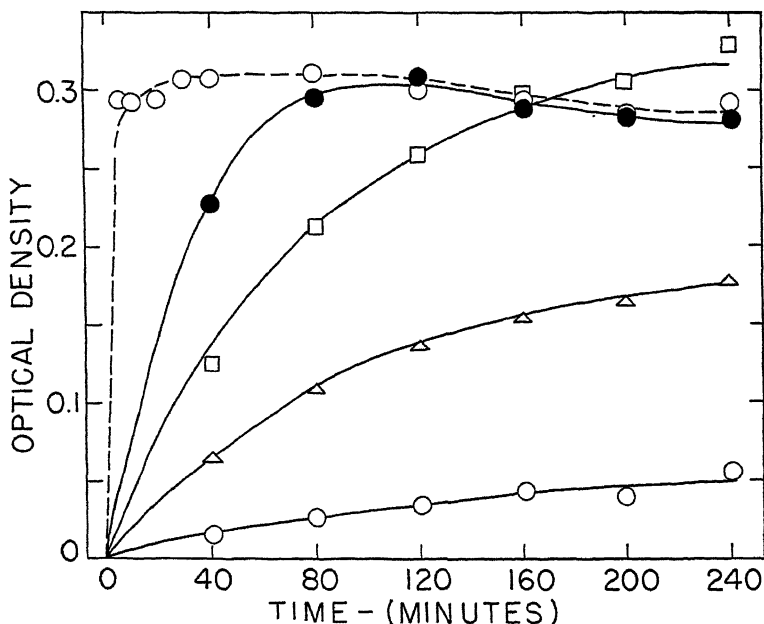


Fig. 1. Response of pyrimidine ribose compounds to modified orcinol reagent. The experimental conditions are outlined in the text. Ribose --- ○ ---, uridine —●—, uridylic acid —□—, cytidine —△— and cytidylic acid —○—.

Ammonia. Russell's phenol-hypochlorite method (12) was used for the determination of ammonia after distillation. In some instances the spectral changes in the ultra-violet attending deamination were observed (3).

Pentose Determination. Ribose when bound in the pyrimidine nucleosides and nucleotides shows limited response to the orcinol reagent of Mejbaum (13) (see Table I). Even the most reactive compound, uridine, gives only about 10% of the expected color intensity. We have found, however, that an increase in acidity and prolonged

heating will render these compounds orcinol positive. The response is not uniform and the test is semiquantitative only with mixtures of pyrimidine ribose derivatives. It can be used for quantitative determinations, if only one compound is present in the unknown and the same substance is used as reference standard. The new procedure is recommended only for estimation of pyrimidine-bound ribose. It does not supersede the Meibaum method for pentose determination under ordinary circumstances. The experimental conditions used in the two procedures were as follows: *Meibaum orcinol test* (13). Reagent: conc. HCl containing 0.1% FeCl₃ and 1.0% orcinol. Procedure: To 1.5 ml. of reagent, 1.5 ml. water solution of the sample is added and the mixture is heated in a boiling water bath for 40 min. (14). After cooling, the volume is adjusted to 5.0 ml. with water. *Modified orcinol test*. Reagent: same as above. Procedure: To the sample in 1.0 ml. of water add 2.5 ml. conc. HCl and 1.5 ml. of reagent. Heat in a boiling water bath for 240 min. Readjust volume to 5.0 ml. with water. Some of the acid is lost during the process of heating. The acidity changes from about 9 N to 8 N. Figure 1 shows the development of color. The absorption maximum is located at 670 mμ as in the Meibaum modification.

Five-ml. volumetric flasks proved satisfactory for both procedures. The color obtained is stable. Some commercial samples of orcinol contain impurities which cause failures. Recrystallization from chloroform is advisable. For routine tests a Klett photoelectric colorimeter (filter 66) was used; for spectrophotometry, a Beckman instrument, model D.

Correct choice of the orcinol method makes possible the following measurements:

(a) Quantitative determination of pyrimidine ribose nucleosides or nucleotides with the same material as reference (modified procedure).

(b) Check for completeness of metabolism of pyrimidine-bound ribose in enzyme systems (modified procedure).

(c) Distinction between pyrimidine-bound and other ribose derivatives (purine-bound ribose, ribose phosphoric acid esters, free ribose) by a combination of the two procedures.

RESULTS

It was observed that washed cells of *Escherichia coli* and *Aerobacter aerogenes* metabolize the carbohydrate of extraneous pyrimidine ribose nucleosides and nucleotides (see Table II). Deamination took place simultaneously. The amounts metabolized exceeded the ribose content of the cells manyfold on a molar basis, and there was no interference with the pentose tests, since the endogenous ribonucleic acid did not diffuse out of the cells during the incubation.

The pH optimum for ribose metabolism was found to be 7.0 with fresh cells. There is a gradual decline of activity at higher pH and a rapid decrease below pH 6.5.

The disappearance of ribose from the incubation mixture is not due to retention of it in the bacterial cell. This was ascertained by orcinol tests of samples without removal of the bacterial cells. The amount of

ammonia formed was less than expected from the ribose metabolism. It appears that part of it is retained by the cells.

The pyrimidine nucleus was not metabolized under our experimental conditions except for the deamination of the cytosine derivatives. The accumulation of uracil was demonstrated by spectrophotometry (see Fig. 2). The absorption maximum shifted during the incubation from 266 $m\mu$ (cytidine) and 268 $m\mu$ (cytidylic acid) to 258 $m\mu$, and that of uridine from 261 $m\mu$ to 258 $m\mu$ which indicates formation of uracil in all instances. The absorption maximum of uracil depends on the acidity. In phosphate buffer of pH 7.0 we found $E_m = 8.3 \times 10^3$. The discrepancy between this value and the absorption intensity observed after incubation shows that a small part of the uracil is either retained by the cells or metabolized.

TABLE II

Metabolism of Pyrimidine Ribosides by E. coli and A. aerogenes

Experimental conditions: 17 mg. of fresh cells/ml.; 0.03 M phosphate, pH 7.0; incubation at 37° for 4 hr. Cells removed by centrifugation, samples of the supernatant analyzed. Pentose determination by the modified orcinol test; ammonia determination in distilled samples (12). The values are corrected for endogenous ammonia formation (0.5 μg .-moles/ml. for *E. coli* and 1.6 μg .-moles for *A. aerogenes*). The endogenous ribose metabolism remained below 0.3 μg .-moles in all instances.

| Compound examined | Concentration used | Ribose metabolized | | Deamination | |
|-------------------|---------------------|--------------------|---------------------|----------------|---------------------|
| | | <i>E. coli</i> | <i>A. aerogenes</i> | <i>E. coli</i> | <i>A. aerogenes</i> |
| | μg .-moles/ml. | % | % | % | % |
| Cytidine | 2.85 | 95 | 97 | 72 | 76 |
| Uridine | 3.4 | 100 | 92 | — | — |
| Cytidylic acid | 2.6 | 89 | 85 | 79 | 83 |
| Uridylic acid | 2.6 | 100 | — | — | — |

This experiment does not permit a decision as to whether deamination occurs at the nucleoside stage or after liberation of cytosine. The literature on this point is conflicting (3,15). Our observations on pyrimidine nucleosidase reported below seem to indicate deamination of cytidine.

Experiments with the Warburg respirometer showed that the ribose is oxidized to carbon dioxide and water. The oxidation, however, is not yet complete at the time when no more response with the orcinol test

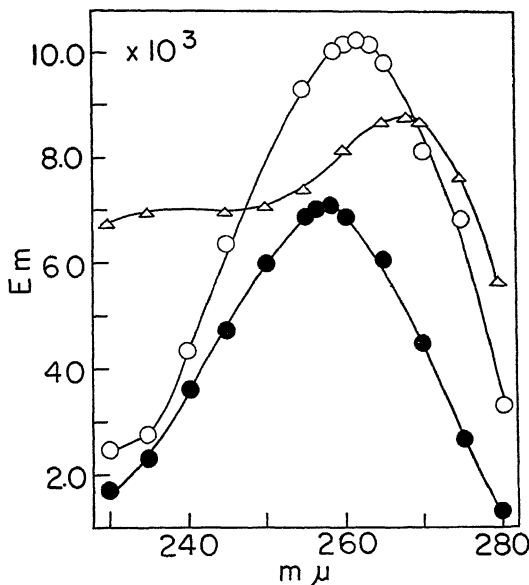


Fig. 2. Spectrophotometric observation of pyrimidine riboside metabolism. Experimental conditions: substrate concentration, 2.85 $\mu\text{g.}$ -moles/ml.; 17 mg./ml. of fresh cells (*E. coli*); 0.1 *M* phosphate, pH 7.0; $t = 37^\circ$. Removal of cells by centrifugation. Uridine -- \circ -- \circ -- (at time 0), -- \bullet -- \bullet -- (after 4 hr.); cytidylic acid -- \triangle -- \triangle -- (at time 0). The absorption spectrum of cytidine at time 0 was almost identical with that of cytidylic acid; after 4 hr. incubation it approached very closely that of the uridine incubate.

is obtained. This indicates temporary accumulation of orcinol-negative intermediates.

To arrest the pyrimidine riboside degradation in an early stage and to demonstrate phosphorylation, experiments with acetone-treated cells were carried out.

Role of Phosphate

The role of phosphate ion in the metabolism of purine ribosides has been established by Klein (2), Kalckar (10), and others (16). For pyrimidine riboside metabolism an analogous function of phosphate has not yet been observed.

The data in Table III suggest strongly that the rate of splitting of cytidine and uridine depends on the phosphate concentration. Positive

results with the Meijbaum orcinol test show that the catabolism of the carbohydrate part does not go beyond the pentose stage.

The accumulation of ribose 1-phosphate could be measured with Lowry and Lopez' method for determination of labile phosphoric acid esters as described by Kalckar (10). This is demonstrated in Fig. 3. The identity of our labile phosphoric acid ester with ribose 1-phosphate is suggested by its mode of formation and its lability toward acid. No significant esterification of phosphate could be found with the Fiske and SubbaRow method.

TABLE III

Role of Phosphate in the Enzymatic Splitting of Pyrimidine Ribosides

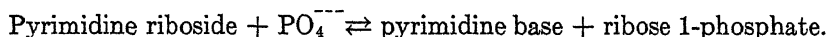
Experimental conditions: 6.6 mg. of acetone-treated cells of *E. coli*/ml.; incubation at 37°, pH 7.0. At the time indicated the samples were deproteinized by trichloroacetic acid.

| Compound examined | Phosphate concentration | Ribose ^a found at time: | |
|------------------------|-------------------------|------------------------------------|----------------|
| | | 0 min. | 90 min. |
| 4.5 μ g.-moles/ml. | <i>M</i> | μ g.-moles | μ g.-moles |
| Cytidine | 0.066 | 0.13 | 3.41 |
| Cytidine | 0.01 | 0.24 | 3.09 |
| Cytidine | H ₂ O | 0.16 | 1.67 |
| Uridine | 0.066 | 1.09 | 3.37 |
| Uridine | 0.01 | 0.88 | 2.91 |
| Uridine | H ₂ O | 0.68 | 1.75 |

^a Determined by Meijbaum orcinol reaction which excludes pyrimidine-bound ribose.

Specificity of the Pyrimidine Nucleosidase

The foregoing experiments show that in acetone-treated cells of *E. coli* the breakdown of pyrimidine ribosides is interrupted after the nucleosidase reaction. The following equilibrium is established:



Attempts were made to test which pyrimidine bases may be components of this equilibrium. For this, uridine was incubated with and without an excess of pyrimidine bases. Only pyrimidine compounds capable of reacting with ribose 1-phosphate may be expected to influence the reaction rate by shifting the equilibrium. To eliminate permeability effects

we used a cell-free nucleosidase preparation. The results are given in Table IV. It may be seen that the excess of uracil retards the splitting of uridine while cytosine and orotic acid are without effect. It appears that in *E. coli* amination and deamination of the pyrimidine nucleus take place only if the latter is combined with ribose. This agrees with the observations of Loring and Pierce (17) on *Neurospora*.

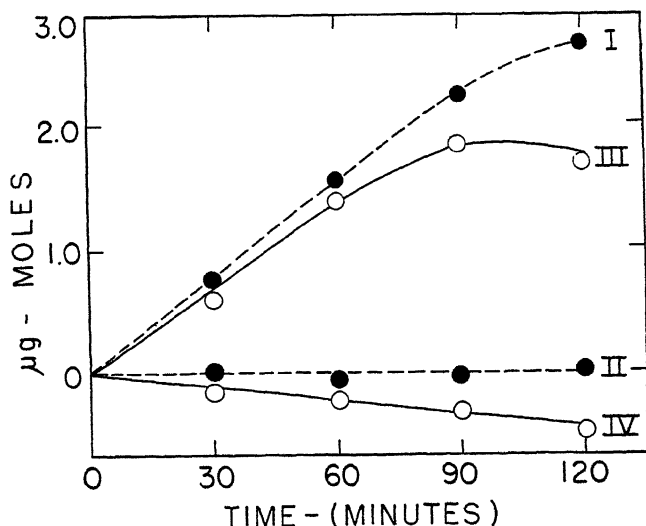


FIG. 3. Formation of ribose 1-phosphate from cytidine. Experimental conditions: substrate concentration, 4.5 $\mu\text{g.}$ -moles/ml.; inorganic phosphate, 0.01 mg.-moles/ml.; acetone-treated cells, 6.6 mg./ml. For analytical procedures consult experimental section.

Curve I: pentose-liberated (Mejbaum orcinol test).

Curve II: control without cytidine (Mejbaum orcinol test).

Curve III: ribose 1-phosphate found.

Curve IV: control without cytidine.

Orotic acid is readily incorporated into the pyrimidine fraction of ribonucleic acid according to Hammarsten and co-workers (18,19,20). The following reaction schemes appear possible:

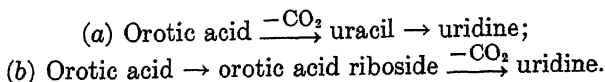


TABLE IV

Influence of Pyrimidine Compounds on Pyrimidine Nucleosidase Reaction

Experimental conditions: Cell-free pyrimidine nucleosidase (0.016 mg. protein/ml.); 0.01 *M* phosphate buffer, pH 7.0; *t* = 37°. The samples were deproteinized with trichloroacetic acid and the splitting of uridine was tested by the orcinol method.

| Compounds examined ^a | 15 min. μg.-moles | Uridine split after: 45 min. μg.-moles |
|-----------------------------------|----------------------|--|
| Uridine (4.5) | 1.27 | 1.47 |
| Uridine (4.5) + uracil (6.7) | 0.62 | 0.97 |
| Uridine (4.5) + cytosine (6.0) | 1.37 | 1.56 |
| Uridine (4.5) + orotic acid (6.7) | 1.23 | 1.42 |

^a Concentration, in parenthesis, in μg.-moles/ml.

To test alternative (a) we incubated orotic acid in Warburg cups with fresh cells, lyophilized cells, acetone-treated cells, and cell-free juice of *E. coli*. In no instance was decarboxylation observed, which seems to rule out scheme (a). The experiment with orotic acid given in Table IV, however, seems to make reaction (b) doubtful. To investigate this point further we prepared ribose 1-phosphate by incubation of uridine with nucleosidase. The esterification was traced by phosphate determination according to Lowry and Lopez (11). The phosphate uptake had come to an end after 40 min. and the concentration of ester was 0.82 μg.-moles/ml. At this time orotic acid to a concentration of 8.0 μg.-moles/ml. was added. Twenty minutes later the concentration of ribose 1-phosphate was 0.81 μg.-moles/ml. Orotic acid, therefore, had not changed the equilibrium.

DISCUSSION

The experiments reported here demonstrate the suitability of bacterial cells for the study of pyrimidine riboside metabolism. Aside from high enzymatic activity the compounds studied seem to be readily diffusible. It may be that the pyrimidine nucleotides undergo dephosphorylation by enzymes on the bacterial surface prior to entering the cell.

A close resemblance of pyrimidine riboside metabolism with that of purine ribosides has been found in this study. The formation of ribose 1-phosphate in the course of pyrimidine splitting had been assumed (21) but not verified earlier. Our study does not seem to support the contention of Klein (2) that enzymatic resolution of the pyrimidine ribose bond is a slow process which resembles the stability of this linkage

toward acid. The action of our bacterial pyrimidine riboside phosphorylase appears to be restricted to uridine. The failure of cytosine to combine with ribose 1-phosphate is in analogy to the inertia of adenine.

Of special interest is the role of orotic acid in pyrimidine nucleoside metabolism. All studies have left open to question whether it is decarboxylated before or after combination with the carbohydrate. Our attempts to demonstrate direct decarboxylation of orotic acid to uracil have failed. On the basis of the experiments of Hammarsten and his group (18,19,20) indicating the rapid incorporation of orotic acid into the pyrimidine nucleotide fraction of ribonucleic acid, we expected formation of orotic acid riboside. This, however, did not occur and further experiments are required to show the significance of this compound. The observations of various laboratories do not agree (19,22) and a difference in the pathways of pyrimidine riboside synthesis in various cells such as *Neurospora*, bacteria, and tissues has to be suspected.

SUMMARY

1. Pyrimidine-bound ribose cannot be determined by Meijbaum's orcinol test. A modified orcinol reaction for this purpose was developed and used in the study of pyrimidine riboside metabolism.

2. Pyrimidine ribosides are split by enzymes from *Escherichia coli* and other organisms. Uracil is accumulated while the carbohydrate is metabolized rapidly. With acetone-treated cells, intermediates of ribose metabolism can be traced.

3. Pyrimidine nucleosidase is a phosphorolytic enzyme. Ribose 1-phosphate is formed from uridine. The role of cytosine and orotic acid in this system is discussed.

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Prolonged Survival of Hyperthyroid Rats Fed Penicillin and Aureomycin Residues¹

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INTRODUCTION

Considerable data are available indicating that the survival time of hyperthyroid rats is dependent in considerable degree on the composition of the diet employed. When immature rats were fed purified rations containing the B vitamins as synthetic factors, the administration of massive doses of thyroid resulted in a number of early deaths, apparently due to cardiac failure (1). Length of survival was significantly prolonged by the concurrent feeding of liver (2) or yeast (1,2); supplements of casein, salt mixture, or the known vitamins, however, were without significant effect (2,3). In the present communication data are presented indicating that dried penicillin mycelia and a fermentation product derived from cultures of *Streptomyces aureofaciens* are also effective in prolonging survival of immature hyperthyroid rats.

EXPERIMENTAL

The basal ration employed in the present experiment consisted of sucrose, 73.0%; casein,² 22.0%; salt mixture,³ 4.5%; and U. S. P. desiccated thyroid,⁴ 0.5%. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 72 mg.; riboflavin, 9 mg.; pyridoxine hydrochloride, 15 mg.; calcium pantothenate, 67.2 mg.; nicotinic acid, 60 mg.; 2-methyl-naphthoquinone, 5 mg.; and

¹ Communication No. 267 from the Department of Biochemistry and Nutrition, University of Southern California.

² Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

³ Hubbel, Mendel, and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

⁴ Thyroid Powder, U. S. P., Armour and Co., Chicago, Ill.

TABLE I

Effects of Dietary Supplements on the Body and Gonadal Weight and Length of Survival of Hyperthyroid Rats

(The values in parentheses indicate the number of animals which survived and on which averages are based.)

| Supplements fed with basal ration | Number of animals | Initial body wt. | Average gain in body wt. on following days of expt. | | Rate surviving ^a | Average survival time ^b | Ovarian wt. | Ventricular wt. | Adrenal wt. |
|---|-------------------|------------------|---|-------------------|-----------------------------|------------------------------------|-------------|-----------------|-------------|
| | | | 28th | 60th | | | | | |
| None | 12 | g. 47.8 | g. 44.3 (10) | g. 64.0 (1) | % 8.3 | days 32.4 ± 4.3 | mg. 18.2 | mg. 700 | mg. 28.2 |
| Aureomycin HCl (100 mg./kg. of diet) | 8 | 46.4 | 49.3 (5) | — | 0.0 | 27.4 ± 5.4 | — | — | — |
| Aureomycin HCl (500 mg./kg. of diet) | 8 | 46.4 | 46.7 (4) | — | 0.0 | 27.5 ± 2.9 | — | — | — |
| Synthetic vitamin mixture | 8 | 46.9 | 75.7 (5) | 89.0 (1) | 12.5 | 41.1 ± 5.1 | 13.6 | 930 | 42.4 |
| Dried penicillin mycelia | 8 | 47.0 | 74.6 (8) | 89.5 (8) | 100.0 | 60.0 ± 0.0 | 16.6 | 817 | 52.5 |
| Aureomycin mash | 8 | 43.8 | 103.0 (8) | 144.8 (5) | 62.5 | 56.6 ± 2.5 | 60.6 | 1066 | 78.7 |
| Extracted Liver Residue | 8 | 45.9 | 95.4 (7) | 130.7 (6) | 75.0 | 53.4 ± 3.9 | 44.3 | 944 | 58.2 |
| Basal ration without thyroid | 10 | 44.0 | 91.9 (10) | 137.4 (10) | 100.0 | 60.0 ± 0.0 | 44.1 | 614 | 41.7 |

^a Experimental period of 60 days.

^b Averages were computed on the basis of a 60-day survival time for animals alive at the termination of the experiment. It is apparent, therefore, that the protective effect of dried penicillin mycelia, aureomycin mash, and liver residue on length of survival was actually greater than indicated in Table I since the majority of animals fed these supplements were still alive at the termination of the experiment.

choline chloride, 1.2 g.⁵ Each rat also received three times weekly the following supplement: cottonseed oil (Wesson) 500 mg., α -tocopherol acetate 1.5 mg., and a vitamin A-D concentrate containing 50 U. S. P. units of vitamin A and 5 U. S. P. units of vitamin D.⁶ In addition to the basal ration the following diets were also employed, consisting of basal ration plus each of the following supplements: (a) 100 mg. aureomycin HCl ⁷/kg. diet, (b) 500 mg. aureomycin HCl/kg. diet, (c) supplements of the

⁵ In view of the increased requirements for thiamine, pyridoxine, and pantothenic acid in the hyperthyroid rat (12), these vitamins were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

⁶ Nopco Fish Oil Concentrate, assaying 800,000 U. S. P. units of vitamin A/g. and 80,000 U. S. P. units of vitamin D/g.

⁷ We are indebted to Dr. E. L. R. Stokstad of the Lederle Laboratories, Pearl River, New York, for the crystalline aureomycin HCl and the aureomycin mash (APF-5) employed in the present experiment. The aureomycin mash was a fermentation product derived from cultures of *Streptomyces aureofaciens* containing approximately 5 mg. aureomycin/g.

following vitamins per kilogram of diet: thiamine hydrochloride, 20 mg.; riboflavin, 20 mg.; pyridoxine hydrochloride, 20 mg.; calcium pantothenate, 60 mg.; nicotinic acid, 60 mg.; biotin, 4 mg.; folic acid, 10 mg.; *p*-aminobenzoic acid, 400 mg.; inositol, 800 mg.; 2-methyl-naphthoquinone, 10 mg.; and vitamin B₁₂,⁸ 30 μ g., (d) 10% aureomycin mash,⁷ and (e) 10% dried penicillin mycelia.⁹ Supplements were added in place of an equal amount of sucrose. In addition to the above rations, two control diets were also tested. These consisted of (a) basal ration plus 10% Extracted Liver Residue¹⁰ and (b) basal ration with thyroid omitted and replaced by an equal amount of sucrose. Previous findings indicate that Extracted Liver Residue contains one or more factors distinct from any of the known nutrients which are effective in prolonging survival and counteracting the retardation in growth and inhibition of gonadal development of immature rats fed massive doses of thyroid (2-5). In the present study the effects of feeding the supplements indicated above were contrasted with that obtained with Extracted Liver Residue in the immature hyperthyroid rat. Seventy female rats of the Long-Evans strain were selected at 21-23 days of age and an average weight of 46.3 g. for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib.* the diets indicated above. Feeding was continued for 60 days or until death, whichever occurred sooner. Animals were autopsied after the 60th day of feeding, and ovarian, adrenal, and ventricular weights determined. Ovaries were fixed in 10% formol and sections prepared and stained with hematoxylin and eosin. Results are summarized in Table I.

RESULTS

Findings indicate that the average length of survival of thyroid-fed rats differed significantly on the various diets employed. Eleven of the 12 rats fed the basal ration succumbed during the experimental period of 60 days (average survival time 32.4 days). Length of survival was significantly prolonged on diets containing dried penicillin mycelia, aureomycin mash, or liver residue. Supplements of all the known B vitamins, however, had no significant effect. In view of the findings of Stokstad *et al.* that crystalline aureomycin HCl promotes growth in the chick (6) and pig (7), tests were conducted to determine the effects of this material on the survival time of thyroid-fed rats. Supplements of aureomycin HCl at levels of 100 mg. or 500 mg./kg. of diet had no significant effect, however, on the length of survival of hyperthyroid rats. In agreement with earlier findings gain in body weight was markedly reduced in animals fed the basal ration with ovaries remaining

⁸ Cobione (Crystalline Vitamin B₁₂, Merck), Merck and Co., Rahway, New Jersey.

⁹ Dried Penicillin Mycelia, kindly provided by Eli Lilly and Co., Indianapolis, Ind.

¹⁰ Extracted Liver Residue, Wilson Laboratories, Chicago, Ill. This liver fraction consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

infantile both in weight and microscopic appearance. Both effects, *i.e.*, retardation in growth and inhibition of ovarian development, were completely counteracted by the administration of liver residue or aureomycin mash. Some increases in body weight over the basal ration was observed during the first 4 weeks of feeding in animals receiving the B vitamin supplement although the increment in body weight was less than that obtained with liver residue or aureomycin mash; subsequently, surviving animals reached a plateau in weight. Ovaries in this group remained infantile both in weight and microscopic appearance. As previously reported aureomycin HCl at levels of 100 mg. or 500 mg./kg. of diet was without significant effect on the growth of hyperthyroid rats (8). Findings with the dried penicillin mycelia were at variance with those obtained with the liver residue or aureomycin mash. Although this supplement was effective in prolonging survival under conditions of the present experiment, it had little if any effect on rate of growth, while ovaries remained infantile both in weight and microscopic appearance. A marked increase in ventricular and, in some cases, adrenal weight was observed in thyroid-fed rats proportional in general to the body weight. The increase in adrenal weight was particularly marked in animals fed the aureomycin mash.

DISCUSSION

Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal (9). This is particularly true for some of the B vitamins. An increased requirement for thiamine (10,11), pyridoxine (12), pantothenic acid (12), folic acid (13), and more recently vitamin B₁₂ (14,15) has been demonstrated following the administration of large doses of thyroactive substances in the rat. In addition to the above, requirements are increased for at least one additional factor as well. Whole liver and its water-insoluble residue has been found to prolong survival and counteract the retardation in growth and inhibition of ovarian development of immature rats fed massive doses of thyroid when casein is the dietary protein and sucrose the dietary carbohydrate (2,16,17). Supplements of all the known nutrients, however, had little if any effect. There is some question whether the factor which prolongs survival is identical to that which promotes growth or gonadal development. Yeast and whole liver, for example, were both effective in prolonging survival when fed at a 10% level in

the diet although yeast in contrast to liver had no significant effect on gonadal development or rate of growth. Whole liver, on the other hand, when fed at a 2% or 4% level in the diet had a pronounced effect on growth but no significant effect on length of survival (17). These findings would seem to indicate that prolonged survival and increased growth were not due to different concentrations of the same factor.

Results of the present experiment indicate that dried penicillin mycelia and aureomycin mash were similar to yeast and liver residue, respectively, in their effects on the hyperthyroid rat. Both dried penicillin mycelia and yeast prolonged survival but had little if any effect on growth or gonadal development; liver residue and aureomycin mash on the other hand not only prolonged survival but promoted growth and ovarian development as well.

SUMMARY

Supplements of dried penicillin mycelia and aureomycin mash prolonged significantly the average length of survival of immature rats fed purified rations containing massive doses of thyroid. Crystalline aureomycin HCl and supplements of all the known B vitamins were without significant effect.

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Antibiotics and Early Growth of Rats Fed a Soybean Oil Meal Diet^{1, 2}

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INTRODUCTION

The failure of many workers to raise rats through successive generations on diets containing protein of vegetable origin or from a purified source was an important consideration in the development of the present concept of animal protein factor (APF) and vitamin B₁₂. Mapson (1) and Russell (2) in 1932 and Folley *et al.* (3) in 1947 indicated that a factor present in fresh liver, whole milk, and similar products would restore normal reproduction and growth in rats fed extracted casein or a vegetable concentrate as protein sources. In light of present knowledge, this response may be ascribed to the presence of vitamin B₁₂ in these animal products. When vitamin B₁₂ became available, it was fed to rats receiving diets based on soybean oil meal or a purified source of protein. Significant increases in post-weaning growth rate of rats were reported when vitamin B₁₂ was added to B₁₂-deficient diets (4,5). Vitamin B₁₂ also restored normal growth in rats made hyperthyroid by the feeding of thyroid tissue (6).

Subsequent experiments with swine and chicks, however, indicated that a factor or factors other than vitamin B₁₂ and known vitamins were required to promote optimal growth. Cunha *et al.* (7) reported that an APF fermentation concentrate produced better growth of pigs on a corn-peanut meal diet than did vitamin B₁₂, and that methionine gave a response when added to the B₁₂-supplemented diet but not when added to the APF-supplemented diet. When it was noted that APF

¹ This investigation was supported in part by funds provided for biological and medical research by the State of Washington, Initiative 171.

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concentrates produced from antibiotic fermentations stimulated growth beyond vitamin B₁₂, the antibiotics themselves were investigated. Aureomycin (8) and streptomycin (9) were shown to increase the growth rate of young pigs on vegetable protein diets.

The object of the present experiment was to determine the growth-promoting effect of crystalline antibiotics for rats fed a diet, the protein of which was supplied by soybean oil meal.

EXPERIMENTAL

Rats of the Long-Evans strain were used in these feeding trials. Females were maintained on the stock colony ration, slightly modified from that of Hawk *et al.* (10), during the mating period. Within 24 hr. after birth of the litters, the females were placed on the soybean oil meal diets shown in Tables I and II. Litters were adjusted to eight pups within 2 days after birth and were weaned at 21 days of age.

TABLE I

Basal Diet and Supplements

| | % |
|---|-------|
| Soybean oil meal (solvent-extracted) | 51.9 |
| Salt mix ^a | 2.0 |
| Glucose | 37.7 |
| Choline | 0.02 |
| Vitamins ^b | 0.025 |
| Soybean oil | 8.0 |
| Vitamins A and D concentrate (5,000 international units A/g., 1,000 units D/g.) | 0.355 |

^a Hubbell *et al.* (11).

^b Vitamins in milligrams: thiamine, 120; pyridoxine, 120; riboflavin, 240; calcium pantothenate, 800; inositol, 240; niacin, 480; *p*-aminobenzoic acid, 1,300; biotin, 3, and folic acid, 24; 0.25 g. of the resulting mixture was included in each kilogram of diet.

The soybean oil meal in this diet supplied 23% protein. The level of known nutrients was judged to be adequate for optimal growth of the rat. In a previous experiment, the addition of 1.1% methionine failed to improve the rate of growth. For this reason, methionine was not added to the diets fed in this experiment.

After weaning, the young rats were distributed at random into groups and fed the diets shown in Table II. They were allowed to feed and drink *ad lib.* for the 21-day experimental period.

RESULTS

The weights of the young rats at weaning and the weight changes of the females during the lactation periods are shown in Table II. Although

the addition of vitamin B₁₂ to the basal diet did not materially increase the weight of the young, it did reduce the weight loss of the female. Streptomycin and the APF concentrate further reduced the weight loss of the females. The latter resulted in a gain of weight and significantly increased the weaning weight of the young rats, as compared with the diet supplemented with vitamin B₁₂ only.

The mean weekly gains of the weanling rats during the 21-day feeding trial are given in Table III. The diets fed to the nursing females are shown in the first column, and the diets eaten by the young rats after weaning are listed in the second column. The last column shows the mean weekly gain per rat. In every case the gain of the young, both

TABLE II
Gain of Young During Nursing Period

| Group | Supplement to basal diet | Number of females | Mean wt. change of females | Number of young | | Mean weaning wt. of young | <i>t</i> |
|-------|---|-------------------|----------------------------|-----------------|--------|---------------------------|-------------------|
| | | | | Started | Weaned | | |
| | <i>per kg.</i> | | <i>g.</i> | | | <i>g.</i> | |
| 1 | None | 6 | -28 | 48 | 48 | 35 | |
| 2 | 100 µg. B ₁₂ | 4 | -11 | 32 | 32 | 37 | |
| 3 | 100 µg. B ₁₂ + 25 mg. streptomycin | 5 | - 6 | 40 | 40 | 42 | 3.55 ^a |
| 4 | 100 µg. B ₁₂ + 5 g. aureomycin APF concentrate (525 µg. aureomycin/g.) | 4 | + 5 | 32 | 32 | 44 | 4.68 ^a |

^a Significant at the 1% level when compared with Group 2.

male and female, was greater when they received supplements of vitamin B₁₂ plus antibiotic than when they were fed the diet supplemented with B₁₂ only. Although these differences in gain are not statistically significant, there is a marked trend of more rapid weight gain in the rats which received antibiotic as well as vitamin B₁₂ in the diet.

It is interesting to compare the gains of the young rats which were from females fed antibiotics during the nursing period and which were then given the diet supplemented with B₁₂ but not antibiotics, groups 3 and 5, with those continued on antibiotics, groups 4 and 6. The rates of gain of groups 3 and 5 fell behind those of 4 and 6, thereby indicating that the effect of the antibiotic does not last after the antibiotic is no

TABLE III
Post-Weaning Gains

| Supplements to basal diet | | Group no. | Mean weekly gain per rat ^a | |
|---|---|-----------|---------------------------------------|---------------------|
| During nursing | After weaning | | Male | Female |
| <i>per kg.</i> | <i>per kg.</i> | | | |
| 100 µg. Vitamin B ₁₂ | 100 µg. vitamin B ₁₂ | 1 | 28 (7) | 21 (8) ^a |
| 100 µg. Vitamin B ₁₂ | B ₁₂ + 5 g. aureomycin concentrate | 2 | 33 (8) | 24 (6) |
| 100 µg. B ₁₂ + 5 g. aureomycin concentrate | 100 µg. B ₁₂ | 3 | 34 (6) | 23 (11) |
| 100 µg. B ₁₂ + 5 g. aureomycin concentrate | B ₁₂ + 5 g. aureomycin concentrate | 4 | 36 (7) | 26 (7) |
| 100 µg. B ₁₂ + 25 mg. streptomycin | 100 µg. B ₁₂ | 5 | 32 (8) | 23 (10) |
| 100 µg. B ₁₂ + 25 mg. streptomycin | B ₁₂ + 25 mg. terramycin | 6 | 34 (9) | 27 (7) |

^a Difference between these groups was not statistically significant.

^b Numbers in parentheses refers to number of rats.

longer made available to the rat. Table III also shows that the young of groups 1 and 2, from females on the diet supplemented with vitamin B₁₂ only grew less rapidly than those from females fed the diets containing antibiotic. Group 1 may be compared with groups 3 and 5, and group 2 with 4 and 6. This difference may be caused by the fact that the young from females fed the antibiotics were larger than those from females fed B₁₂ without antibiotic.

TABLE IV
Post-Weaning Gains of Vitamin B₁₂-Deficient Rats

| Lot | Supplements to basal diet <i>per kg.</i> | Mean weekly gain <i>g.</i> |
|-------------------------|---|-------------------------------|
| 1 Albino | None | 17 |
| | 100 µg. vitamin B ₁₂ | 29 |
| | 100 µg. B ₁₂ + 5 g. aureomycin concentrate | 34 |
| | 100 µg. B ₁₂ + 5 g. streptomycin concentrate | 33 |
| 2 Long-Evans rats | None | 11 |
| | 100 µg. vitamin B ₁₂ | 27 |
| | 100 µg. B ₁₂ + 5 g. aureomycin concentrate | 24 |
| | 100 µg. B ₁₂ + 25 mg. streptomycin | 28 |

A feeding trial had been previously run in which all females were fed the vitamin B₁₂-deficient diet during nursing so that their young were B₁₂ deficient. After weaning, the young rats were fed the diets shown in Table IV. The mean weekly gains per rat are also shown in Table IV. In lot 1, using albino rats, there was a trend for the antibiotic-supplemented rats to gain faster than those supplemented with vitamin B₁₂ only. In lot 2, where Long-Evans rats were used, the young supplemented with vitamin B₁₂ only gained as rapidly as those which received antibiotic as well. This was the only experiment in which antibiotics failed to give a response over vitamin B₁₂. There was, however, the usual marked response of the vitamin B₁₂-supplemented group over the B₁₂-deficient group.

DISCUSSION

The data obtained in these feeding trials indicate that the antibiotics, terramycin, streptomycin, and aureomycin (the latter supplied by the APF concentrate) stimulate the growth of rats beyond that given by vitamin B₁₂ alone. It is likely that this effect is mediated by the action of the antibiotics on the intestinal flora of the rat. It should be emphasized that these feeding trials were carried out on a semi-purified diet in which glucose was the main source of carbohydrate. Several reports (12-16) have indicated that the inclusion of various carbohydrates in animal diets affects the flora of the gut and that the alterations in bacterial population may be capable of influencing the nutritional requirements and growth of the host animal. Hundley (17) demonstrated that the rat requirement for niacin increased when fructose was the only source of dietary carbohydrate. The interpretation of the feeding trials presented above may be complicated by the combined or antagonistic effects of both the glucose and antibiotic on the intestinal flora of the rat. Subsequent feeding trials are being conducted to investigate the effect of antibiotics on growth when added to a practical diet.

In 1950 Sunde *et al.* (18) reported that chicks fed fish-solubles, or a liver concentrate as additions to practical-type rations, were heavier at the end of the experiment than those fed supplementary vitamin B₁₂. They concluded that fish and liver contain an unknown factor other than vitamin B₁₂. In support of this conclusion, Ershoff (19) found that a liver preparation, which was a known source of vitamin B₁₂, failed to correct thyrotoxicosis in mice, but that a water-insoluble fraction of whole liver would relieve the signs of the hyperthyroid condition. It is

unlikely that the growth factor reported by these workers to be present in fish-solubles and liver is similar to the antibiotics used in the trials reported in this paper. The probable action of fish-solubles and liver was to relieve marginal deficiencies or to correct imbalances in vitamins or amino acids, whereas the effect of the antibiotics was most likely on the intestinal flora.

Further investigations will be required to elucidate more clearly the nature of the additional factor, if any, in liver and fish-solubles.

In a recent publication, Schultze (20) reported that a diet based on soybean oil meal protein, supplemented with methionine and fed at a level to supply 24% protein, supported normal reproduction and satisfactory post-weaning weight gains for four successive generations. This performance was not improved by the addition of sources of vitamin B₁₂ to the diet. This lack of response to B₁₂ is not in agreement with the results of the feeding trials reported above. With the exception of the methionine supplement, previously found to be without effect, the diets of the present investigation and that of Schultze were similar, both having complete vitamin and mineral supplements and having almost the same protein levels, 23 and 24%. However, Schultze used sucrose as the carbohydrate, whereas in this investigation glucose was used. Other workers (12-17) have found that various carbohydrates had different effects on the intestinal flora and the growth requirements of the rat, so that the sucrose in the diet of Schultze may have prevented the response to vitamin B₁₂. There is also the possibility that difference in the strains of rats, as suggested by the data of Table III, may be responsible for the lack of agreement.

ACKNOWLEDGMENTS

Materials which were generously supplied by Lederle Laboratories, Inc., Pearl River, New York; Merck and Co., Inc., Rahway, New Jersey; and Chas. Pfizer & Co., Inc., Brooklyn, New York, were of great assistance in this study.

SUMMARY

Rats of the Long-Evans strain were fed a semipurified basal diet with soybean oil meal as the source of protein, and glucose as the carbohydrate source. This diet was supplemented with minerals and vitamins but was deficient in its vitamin B₁₂ content. Supplementation of this basal diet with vitamin B₁₂ did not increase the weaning weights of young rats, but the weaning weights were significantly increased when

the antibiotics, aureomycin and streptomycin, were added to the basal diet along with vitamin B₁₂. A marked trend was observed in which the post-weaning weight gains of young rats were greater when their diets were supplemented with vitamin B₁₂ and the antibiotics, aureomycin, terramycin, or streptomycin, as compared with the diet supplemented with vitamin B₁₂ only. It is likely that the increase in growth rate is mediated through alterations in the intestinal flora of the rat, and that the carbohydrate source, as well as the antibiotic, is capable of changing the bacterial population of the digestive tract.

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The Metabolism of Radioactive Salicylic Acid *

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INTRODUCTION

Although salicylic acid has been used in the treatment of rheumatic fever since 1876, the possible mechanisms of its action have only recently been indicated.

Coburn has postulated that the antirheumatic effect of salicylic acid may be due to an interference with immune reactions characteristic of the disease (1). Lutwak-Mann has studied the inhibitory effect of salicylate on several enzyme systems (2). Guerra has found that salicylate inhibited the action of hyaluronidase (3) although later work seems to imply that the active substance is a metabolic product of salicylic acid (4,5). Thus the identification of salicylate metabolites becomes a matter of considerable interest.

It is felt that a better understanding of salicylate metabolism, studied using the isotope tracer technique, may help elucidate the underlying mechanisms of rheumatic fever.

EXPERIMENTAL

Salicylic acid labeled with C^{14} in the carboxyl group was synthesized from radioactive carbon dioxide and sodium phenate by a modification of the method of Schmitt (6).

From 1.97 g. (0.01 mole) C^{14} barium carbonate (1.32 mcuries), 97 mg. of salicylic acid, m. p. 155–6.5° was obtained. The yield was 7.0%; unused carbon dioxide was easily recovered. After one recrystallization from water, m. p. 158–9°C. (reported 159°). For a sample of nonisotopic salicylic acid prepared by the same method:

Calcd.: C = 60.87%; H = 4.38%. Found: C = 60.77%; H = 4.40%.

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The activity of the sample was 81,200 counts/min./mg., using a thin-window counter.

For radioactivity measurements, tissues were converted to barium carbonate by wet oxidation (7). Urine and pure organic compounds were plated directly.

Radioactive salicylic acid was administered as the sodium salt to young, mature albino rats by intraperitoneal injection or by stomach tube. Urine and feces were collected, respired carbon dioxide was absorbed in alkali, and blood was obtained following decapitation.

Metabolic products of salicylic acid in plasma and urine were studied by paper chromatography. Aliquots of plasma or urine were acidified, extracted with ether and ethyl acetate, the extracts evaporated to a small volume and placed on a 32×400 mm. strip of Whatman No. 4 filter paper. The chromatogram was developed with 1 part butanol to 1 part pyridine equilibrated with 2 parts of saturated sodium chloride solution (8), by the ascending method (9) (Fig. 1). The aqueous residue from the ether-ethyl acetate extraction was evaporated to dryness *in vacuo*, treated with abso-

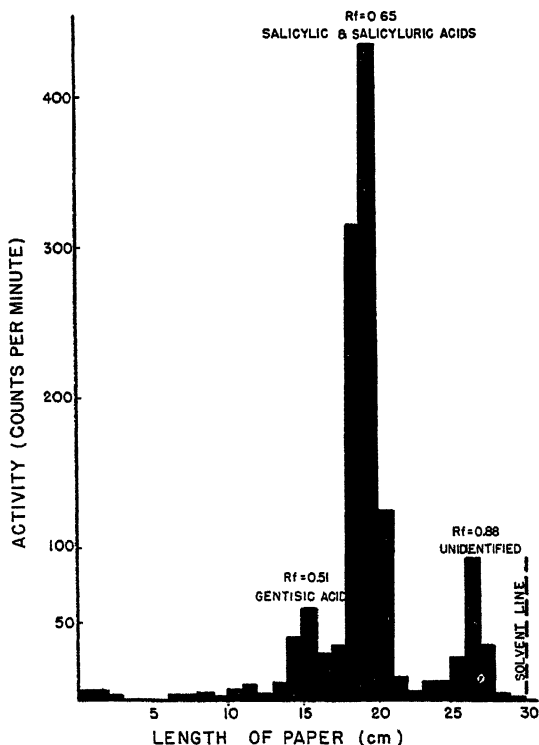


FIG. 1. Radioactivity on paper chromatogram of ether-ethyl acetate extract of rat urine.

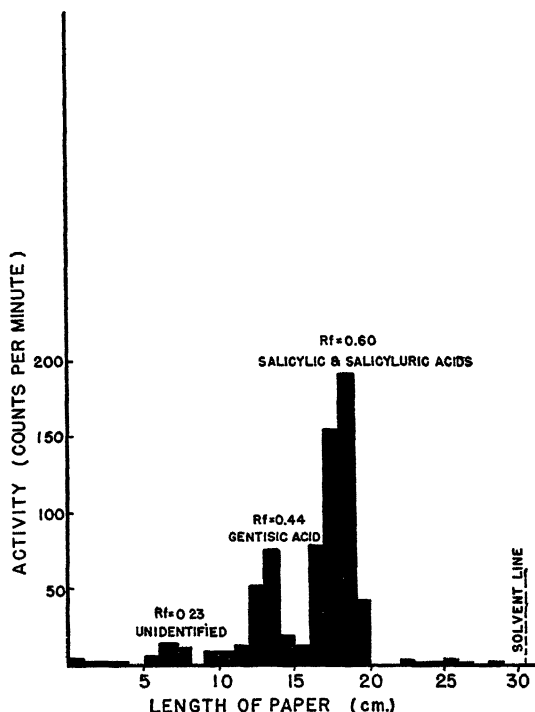


FIG. 2. Radioactivity on paper chromatogram of absolute ethanol extract of dry residue from previous ether-ethyl acetate extraction of rat urine.

lute ethanol to dissolve the more polar metabolites, filtered, and developed as before (Fig. 2). After approximately 16 hr. the paper strips were dried, cut into 1-cm. segments and the radioactivity measured (10).

Identification of plasma and urine metabolites was effected by comparison of experimental R_f values with published values and by isolation after addition of carrier. In the latter procedure, authentic samples were added to extracts of urine and plasma and recrystallized to constant activity. R_f values of substances shown in Fig. 2 are probably displaced by contaminants extracted from the urine residue.

Experiment I

A 199-g. male rat was injected intraperitoneally with 6.5 mg. radioactive salicylic acid. After 24 hr. the rat was decapitated. Distribution of the injected radioactivity in excreta and organs is shown in Table I. The occurrence of urinary metabolites is shown in Figs. 1 and 2.

For identification of the urinary metabolites, the published R_f values of salicylic acid and gentisic acid, 0.65 and 0.51, respectively (8), were found to correspond to

TABLE I

*Distribution of Radioactivity 34 hr. After Intraperitoneal Injection
of Carboxyl-Labeled Sodium Salicylate to Rat*

| | Radio- activity counts/min. | Per cent of total injected |
|--|-----------------------------------|----------------------------------|
| Sodium salicylate injected | 525,000 | 100 |
| Urine | 470,000 | 90 |
| Carbon dioxide | 1,200 | 0.2 |
| Feces | 1,800 ^a | — |
| Blood, liver, kidney, brain, spleen, intestine | 0 | 0 |
| Substances extractable with ether from alkaline urine | 0 | 0 |
| Urinary substances insoluble in ether, ethyl acetate, and alcohol | 0 | 0 |

^a Activity probably due to contamination by urine.

the experimental values. The substances shown in Figs. 1 and 2 having R_f values of 0.23 and 0.88 appear to be conjugates since these two maxima disappear following acid hydrolysis. Salicyluric acid has an R_f value almost identical with salicylic acid and was thus not distinguishable on the paper chromatogram. The presence of both salicylic acid and salicyluric acid in the urine was proved by isolation after the addition of carrier. After two recrystallizations, salicylic acid reached constant activity of 700 counts/min./mg.; after three recrystallizations, salicyluric acid reached constant activity of 40 counts/min./mg.

Experiment II

A 223-g. male rat was injected intraperitoneally with 4.0 mg. of radioactive salicylic acid. After 2 hr. the rat was decapitated and the urine and plasma investigated. The pattern of the urinary metabolites was the same as in Expt. I. The pattern of the plasma metabolites showed the presence of maxima at $R_f = 0.65$ and 0.90. The latter appears to be the only conjugated form of salicylic acid occurring in plasma; however, the presence of this substance in plasma was not confirmed by additional feeding and injection experiments. Salicyluric acid was not present in the plasma, for isolated carrier salicyluric acid was devoid of radioactivity after three recrystallizations.

Experiment III

A 152-g. female rat was fed 2.3 mg. radioactive salicylic acid. After 1 hr. the rat was decapitated and the urine and plasma investigated. The pattern of the urinary metabolites was the same as in Expt. I. The plasma showed the presence of salicylic acid only.

DISCUSSION

This study indicates that salicylic acid administered to rats is not significantly retained in the body but is mostly excreted in the urine

within 24 hr. There is an insignificant amount of carbon dioxide formed from the carboxyl carbon.

Five radioactive compounds have been demonstrated in the urine of rats. These are: salicylic acid, salicyluric acid, gentisic acid, and two unidentified substances. Only gentisic acid has been reported in the rat previously (11). The pattern of the urinary metabolites is the same after feeding or intraperitoneal injection.

Of the known salicylate detoxication products from other species (12), only salicylglucuronic acid, salicyl ethereal sulfate, and uraminosalicylic acid have not yet been identified in rat urine. The compound of $R_f = 0.23$ may be the ethereal sulfate or the glucuronate; however, Lutwak-Mann reports the inability of rats to form a glucuronate (2), and the ethereal sulfate is reported to be very unstable in acid solution (13). Authentic samples of the glucuronates and ethereal sulfate were not obtainable for comparison of R_f but a low value would be expected from their polar character. The compound of $R_f = 0.88$ is either the uraminosalicylic acid or a new metabolite. Since the structure of the uramino compound is unknown, no conjecture can be made of its polarity.

Rat plasma contains salicylic acid although in one experiment the presence of a compound of $R_f 0.90$ was demonstrated.

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SUMMARY

1. Salicylic acid has been synthesized containing C^{14} in the carboxyl group.
2. After administration to rats, most of the radioactivity was excreted in the urine within 24 hr.
3. There is an insignificant conversion of the carboxyl group to carbon dioxide.
4. There is no significant retention of salicylic acid by the body after 24 hr.
5. The urine of rats contains five radioactive substances: salicylic acid, salicyluric acid, gentisic acid, and two unidentified substances.
6. The plasma contains salicylic acid and one unidentified substance.

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The Metabolism of Potato Slices¹

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INTRODUCTION

Investigations on the metabolism and on the mechanisms of respiration in plants have been revived recently, and numerous publications have appeared on the fermentation and oxidation pathways of plant leaves, seeds, and roots. However, similar studies with the potato tuber have been conducted rather haphazardly. The terminal steps in the transfer of electrons from foodstuff to molecular oxygen were debated by Boswell and co-workers (1,2,3), Baker and Nelson (4), and Schade and co-workers (5,6). The relation among respiration, salt absorption, and the synthesis of protein has been studied by Steward and Preston (7). Some work has also been done on the detection of isolated enzyme systems. Hanes (8) found phosphorylase; Kalckar (9), apyrase; Borchardt and Pringsheim (10), α - and β -amylase; Bernheim (11), aldehyde oxidase; Vennesland (12), oxalacetic carboxylase; Levy *et al.* (13), cytochrome oxidase; Chodat and Staub (14), tyrosinase; Stone (15), ascorbic acid oxidase; and Banga and Szent-Györgyi (16), dioxymaleic acid oxidase.

The studies presented here are the outgrowth of a longstanding interest of one of the authors (Link) in the metabolic disturbances of the potato tuber known as "Black Heart." The understanding of the mechanism of this alteration required some knowledge of the metabolic activities of the normal potato.

EXPERIMENTAL

Most of the potato tubers used in these experiments were stored at 11° and were brought to room temperature at least 24 hr. before use to eliminate possible variations

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in gas exchange associated with large changes in temperature. With a cork borer of 1.5 cm. diameter, cylinders were obtained which were sliced (about 0.5 mm. thick) with a thin razor blade and were cut into three portions before being put into the Warburg vessels. They were suspended in 3 ml. phosphate buffer 0.01 *M* (unless otherwise stated), pH 6.0. Substrates were used at a concentration of 0.01 *M*, unless otherwise stated. The temperature of the water bath was ordinarily 28°. Measurement of gas exchange was performed in the usual Warburg-Barcroft manometers and vessels. Determinations were performed as follows: lactic acid [Barker and Summerson (17)]; pyruvic and α -ketoglutaric acids [Friedemann and Haugen (18)]; citrate [Speck *et al.* (19)]; phosphorus [Gomori (20)]; ammonia [Conway (21)]; acetate and butyrate [Friedemann (22)]; glutamic acid [Cohen (23)]; aspartic acid [Braunstein *et al.* (24)]; alanine [Barron and Huggins (25)]; and ethyl alcohol, by a modification of Nicloux's method (26). The reagents used were the dilute solutions of Nicloux. An aliquot of the fluid to be analyzed after protein precipitation with $\text{Zn}(\text{OH})_2$ was introduced into the micro-Kjeldahl distillation apparatus of Ma and Zuazaga (27). After addition of an equal volume of saturated picric acid and of 1 ml. of semicarbazide (0.5 *M* containing 1 *M* sodium acetate), the fluid was steam-distilled into an ice-cold test tube until about 5 ml. was collected. Alcohol was oxidized in 35-ml. glass-stoppered centrifuge tubes. To the 5 ml. of distillate were added 2.5 ml. 50% H_2SO_4 (ice cold) and 1 ml. of the bichromate reagent. The tubes were stoppered and sealed with concentrated H_2SO_4 , then shaken and kept in a water bath at 85° for 1 hr. The excess bichromate was reduced with excess Mohr's salt which was then titrated with permanganate.

Blank analyses were made with the substrate added to potato slices suspended in the appropriate buffer kept in an ice-cold water bath.² At the end of the experiments, the tissue was dried overnight at 105°. Lithium pyruvate, cis-aconitic acid, α -ketoglutaric acid, oxalacetic acid, adenosine triphosphate (ATP), cytochrome c, diphosphopyridine nucleotide (DPN), diphosphothiamine, and C^{14} -carboxyl-labeled acetate and butyrate were prepared at the laboratory. The other reagents were obtained from commercial houses. Measurements of pH values were made with a glass electrode in conjunction with a Cambridge Instrument pH meter. In the experiments with C^{14} -labeled fatty acids, the CO_2 that was trapped in the NaOH (1 *M*) used in the center cups of the Warburg vessels was transferred quantitatively to a centrifuge tube; it was then passed to another centrifuge tube, containing carbonate-free 1 *M* NaOH, by slow addition of HCl (1 *M*) and a current of nitrogen. On addition of barium nitrate, BaCO_3 precipitated; it was washed three times with CO_2 -free distilled water, and then with methanol. The BaCO_3 was dried, weighed, and transferred as usual on to the aluminum discs (1.42 cm.² area). A known amount of carrier BaCO_3 was added so as to have on these discs about 35 mg. of material; this thickness is such as to be considered of "infinite" thickness for the purposes of radioactivity measurements. The counts were made in the Nucleometer, to which was attached a Nuclear Instrument

² The amount of substrates added to potato slices, when kept in an ice-water bath, changed very little from 5 min. after substrate addition to 3 hr. As an example: to 108 mg. dry weight potato slices in 5 ml. of 0.01 *M* phosphate, pH 6.0, was added 3.20 mg. of pyruvic acid as the potassium salt. One hour later it was 3.15 mg.; 2 hr. later, 3.13 mg.; and 3 hr. later, 3.10 mg.

Model 163 Scaling unit. Specific activity, as defined here, is the number of counts per minute of this infinitely thick sample. The volatile acids were steam-distilled in the presence of HgO and titrated. The sodium salts were then converted into silver salts, which were burned in a microcombustion tube containing a roll of platinum gauze.

Respiration of Potato Slices

It has been customary to keep the potato slices under running tap water for long periods of time (15-40 hr.) before measuring the respiration of this tissue. The reason given for such treatment has been that the O_2 uptake as well as the CO_2 production increases under those conditions. It is obvious that such drastic treatment will produce a number of alterations, particularly damage of the cell membrane and loss of water-soluble coenzymes. This customary long washing period was avoided and the experiments were performed shortly after cutting the tissue.

The O_2 uptake of freshly cut potato slices was lower than the values found on washed slices, as previously reported by Boswell and Whiting (1), Stiles and Dent (28), and Schade *et al.* (5,6). The rate of O_2 consumption was constant for 3 hr., the maximum duration of the experiments. The Q_{O_2} was 0.35 (cu. mm./mg. dry weight/hr.) and the Q_{CO_2} , 0.35, giving a respiratory quotient (R. Q.) of 1 (Table I). Potato slices washed in tap water for 24 hr. gave values more than twice those obtained in freshly cut slices; the R. Q., however, varied from 1.2 to 1.6. Definite differences were found in different varieties of potatoes. Thus, Bliss Triumph potato slices gave higher values than McClure potato slices.

Since most of the slices made from a cylinder along a diameter of a tuber consist of tissues from the medulla, the medullary rays, and the

TABLE I

Respiration of Potato Slices in Ringer-Phosphate Buffer, 0.01 M
pH 6.0. Temperature 28°C. Figures give cu. mm./mg. dry weight/hr.
Volume in flasks, 3 ml. Dry weight of slices about 200 mg.

| Experiment no. | O_2 uptake cu. mm. | CO_2 output cu. mm. |
|----------------|-------------------------|--------------------------|
| I | 0.40 | 0.41 |
| II | 0.35 | 0.35 |
| III | 0.33 | 0.34 |
| IV | 0.34 | 0.34 |
| V | 0.36 | 0.35 |
| Average | 0.355 | 0.355 |

internal phloem, and only a small number consist of tissues from the cortex, the pericycle, the outer phloem, and the xylem, the respiratory rates of slices from the ends and from the remainders of such cylinders were compared. There was no appreciable difference; nor was there any difference between newly harvested tubers and tubers which had been kept in storage until they had begun to sprout and shrivel.

The Fermentation of Carbohydrate

In all living cells with few exceptions (some algae, and some bacteria) the metabolism of carbohydrates seems to start with an anaerobic phosphorylative degradation to pyruvic acid, the first phase of fermentation. In the second phase of fermentation, pyruvate either is decarboxylated to acetaldehyde by carboxylase (aldehyde itself being reduced to alcohol by reduced diphosphopyridine nucleotide (DPNH_2) and alcohol dehydrogenase), or is reduced to lactic acid by DPNH_2 and lactic dehydrogenase. Yeast and higher plants seem to utilize mostly the first route, while bacteria and animal tissues utilize the second, *i.e.*, lactic acid formation. It would seem that the fermentation of carbohydrate in potato tuber follows this phosphorylative pathway. In fact, Hanes (8) found *phosphorylase*, the enzyme which initiates the phosphorylation of starch to glucose 1-phosphate.

Indirect evidence of the presence of *hexokinase* in potato slices is presented in Fig. 1. The fermentation of glucose (as determined manometrically in $\text{NaHCO}_3\text{-N}_2\text{:CO}_2$ by CO_2 formation) was inhibited by DL-glyceraldehyde, which is thought to be a specific inhibitor of hexokinase (29). *Zymohexase* (hexose diphosphate \rightleftharpoons aldatriose phosphate + ketotriose phosphate) was found in potato. To the pulp of 1 kg. of potatoes ground in a meat grinder, 1 l. of water was added, and the suspension was kept shaking in the cold room (3°) for 1 hr. Then 8-hydroxyquinoline (100 mg.) was added to diminish melanine formation. The suspension was filtered through muslin, and $(\text{NH}_4)_2\text{SO}_4$ was added to the filtrate, 30 g./100 ml. filtrate. After centrifugation the precipitate was discarded, and 10 g. $(\text{NH}_4)_2\text{SO}_4$ /100 ml. of fluid was added to the supernatant fluid. The precipitate obtained after centrifugation was dissolved in water and was heated to 60° for 2 min. The suspension was filtered and was used for tests of *zymohexase*. To 1 ml. of filtrate were added 1 ml. of 0.1 *M* veronal buffer (pH 8.4) and 0.2 ml. 1 *M* KCN (brought to pH 7.4 with acetic acid). After 10 min. incubation

at 35°, 1 ml. of 0.1 *M* hexose diphosphate (previously warmed to the same temperature) was added rapidly. At the end of 3 min., an equal volume of 10% CCl_3COOH was added to precipitate the proteins. The triose phosphate cyanohydrin formed was estimated in the filtrate after treatment for 20 min. with 1 *N* NaOH to 1 ml. of filtrate. The liberated inorganic phosphate was 100 $\mu\text{g.}/1$ ml. of enzyme (5 mg. protein), i.e., 0.4 mg. of triose phosphate/mg. protein/hr.

As previously stated, pyruvate in the second phase of fermentation may be either decarboxylated to acetaldehyde by carboxylase or reduced to lactic acid by lactic dehydrogenase. Both enzymes were found in potato tuber.

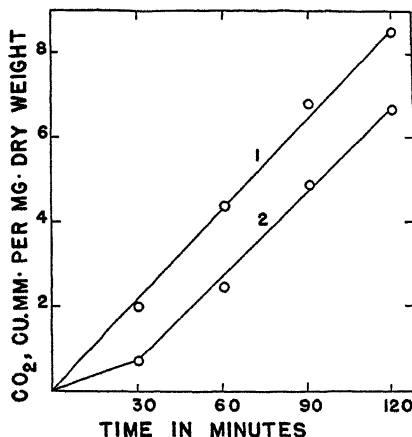


FIG. 1. Effect of DL-glyceraldehyde (0.01 *M*) on the fermentation of glucose by potato slices (CO_2 formation). 1. Control, 2. glyceraldehyde, 0.01 *M*.

Carboxylase was detected as follows. Peeled potatoes were cut into small slices and were ground in the Waring Blender in the presence of a mixture of ice and water (equal volumes). The starch, which was allowed to settle, was discarded, and the supernatant fluid was treated with 10 vol. of cold acetone (-10°). The acetone precipitate was recovered by filtration and was dried in air. The decarboxylation of pyruvate was measured manometrically. The main portion of the Warburg vessel contained 100 mg. of the acetone powder and 3 ml. of a solution containing 0.01 *M* phosphate, pH 6.1, and 0.002 *M* MgCl_2 . The side arm contained 0.3 ml. of 0.01 *M* pyruvate and 0.1 ml. diphosphothiamine

(10 $\mu\text{g.}$). After temperature equilibration, pyruvate was tipped from the side arm and the CO_2 formation was measured; 50 cu. mm. of CO_2 was produced in 30 min., while in the absence of pyruvate there was none (Fig. 2).

Potato *lactic dehydrogenase* was prepared mainly according to Straub's method for the isolation of muscle lactic dehydrogenase (30). Two kilograms of potatoes were peeled and ground in a meat grinder and then squeezed through cloth. The solid portion was shaken with 500 ml. of

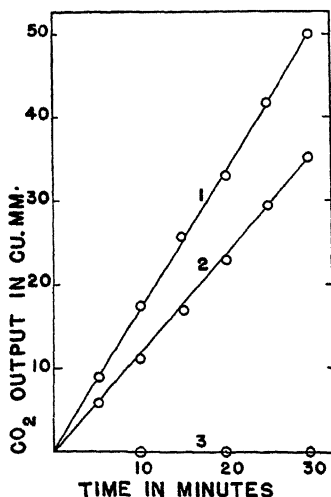


Fig. 2. Carboxylase in potato (acetone-dried preparation). Buffer, phosphate, 0.01 M , pH 6.0. MgCl_2 , 0.005 M . Na pyruvate, 0.01 M . Diphosphothiamine, 10 $\mu\text{g.}$ Temperature 28°C. Volume, 3 ml. 1. Enzyme + diphosphothiamine + pyruvate; 2. enzyme + pyruvate; 3. enzyme alone.

cold distilled water and pressed through cloth again. In this way 1600 ml. of fluid was collected. To this fluid was added about 100 mg. of 8-hydroxyquinoline, and the mixture was left at 3° for 1 hr. to let the starch settle. The fluid was decanted and was shaken for 5 min. with $\text{Ca}_3(\text{PO}_4)_2$ gel [85 ml. containing 8.5 g. $\text{Ca}_3(\text{PO}_4)_2$]. After centrifugation, the supernatant fluid was discarded and the solid was treated with 500 ml. of 0.2 M phosphate buffer, pH 7.2. The mixture was shaken for 10 min. and then to the supernatant fluid was added 200 g. $(\text{NH}_4)_2\text{SO}_4$; the mixture was stirred and then filtered. The precipitate from the filter

paper was dissolved in 10 ml. 0.2 *M* phosphate buffer, pH 7.2, and was centrifuged for 1 hr. at 15,000 r.p.m. The supernatant fluid contained 0.5 mg. protein/ml. To test the presence of lactic dehydrogenase, 0.3 ml. of this fluid (0.15 mg. protein) was used, to which were added 2 ml. of 0.1 *M* phosphate buffer, 0.5 ml. water, 0.3 ml. 1 *M* lithium lactate, and 0.2 ml. 1 *M* KCN (adjusted to pH 7.4). Oxidation of lactate was measured in the Beckman spectrophotometer at 3400 Å. At time 0, 0.2 ml. DPN (1 mg.) was added to the quartz cells and the reduction of DPN was followed (Fig. 3).

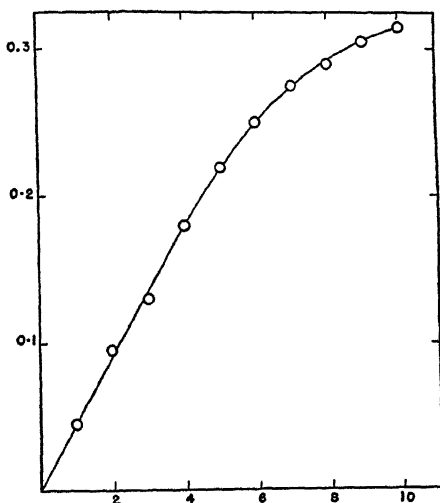


FIG. 3. Oxidation of lactate by potato lactic dehydrogenase. Abscissa, time in minutes; ordinate, *E* value ($\log I_0/I$) at 3400 Å., indication of the rate of reduction of DPN; pH 7.2; temperature 20°.

Little is known about the end products of potato fermentation of glucose. Stoklassa (31) stated that the fermentation produced ethyl alcohol solely, while Kostychew (32) reported finding a $\text{CO}_2:\text{CH}_3\text{CH}_2\text{-OH}$ ratio of 3.6:1 in cut potato tubers.

The anaerobic fermentation of glucose, as measured by CO_2 production in bicarbonate buffer saturated with $\text{N}_2:\text{CO}_2$ (95:5), was found to be maximum at pH 7.0. The pH value in these experiments was controlled by changing bicarbonate concentration (Table II). The CO_2 produced in these experiments may have come from alcohol ferment-

TABLE II

Influence of pH on Fermentation of Carbohydrate by Potato Slices

Glucose, 0.01 *M*. Buffer, bicarbonate:CO₂ or Ringer-bicarbonate:CO₂, 3 ml. Gas phase, N₂:CO₂(95:5). Temperature 28°C. Figures give cu. mm. production/mg. dry weight/hr.

| pH | Buffer | CO ₂ cu. mm. |
|-----|------------------------------------|----------------------------|
| 6.2 | Bicarbonate:CO ₂ | 0.06 |
| 6.2 | Ringer-bicarbonate:CO ₂ | 0.11 |
| 7.0 | Ringer-bicarbonate:CO ₂ | 0.40 |
| 7.4 | Ringer-bicarbonate:CO ₂ | 0.36 |

tation ($C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$), from lactic acid fermentation ($C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$; $2CH_3CHOHCOOH + 2NaHCO_3 \rightarrow 2CH_3CHOHCOONa + 2CO_2 + 2H_2O$), or from both processes, since the enzymes carboxylase and lactic dehydrogenase were both found in potato.

To determine the end products of carbohydrate fermentation in potato slices, the experiments were performed at pH 7.4 in Ringer-bicarbonate (5 ml.) with N₂:CO₂ as the gas phase. The vessels were incubated for 2 hr. at 28°. The CO₂ production (average value given by 4 vessels) was 179 cu. mm. The amount of alcohol formed was 161 cu. mm. (332 μg.); lactic acid was 9.5 cu. mm. (38 μg.) (Fig. 4). Thus, glucose fermentation in these potato slices must have proceeded through both pathways, the molar ratio of alcohol:lactic acid being 17:1.

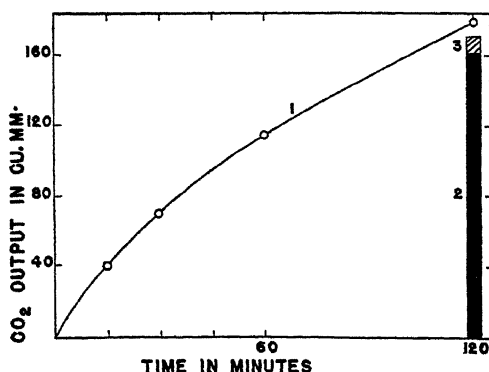


FIG. 4. Fermentation of glucose by potato slices. Buffer, Ringer-bicarbonate, 1 *M*, pH 7.0, volume 3 ml. Gas phase, N₂: CO₂ (95:5). Glucose, 0.01 *M*., temperature, 28°C. Dry weight of potato slices, 366.1 mg. Per cent CO₂ accounted for, 95.3. 1. CO₂ production; 2. alcohol formation; 3. lactic acid formation.

The Oxidative Pathway of Carbohydrate Metabolism

a) *Pyruvate*. In the presence of oxygen the pyruvate formed during the first phase of fermentation may be oxidized directly without going through alcohol and lactic acid. Pyruvate decarboxylated to acetaldehyde by carboxylase will be oxidized by aldehyde oxidase to acetate [Bernheim (11)]; pyruvate may be oxidized directly to acetate, or it may be metabolized by dismutation (30), ($2\text{CH}_3\text{COCOOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{-CHOHCOOH} + \text{CH}_3\text{COOH} + \text{CO}_2$). An indication of the relative importance of these pathways may be obtained by measuring pyruvate metabolism in the presence and in the absence of oxygen: dismutation and decarboxylation alone will proceed in the absence of oxygen, and the value of the oxidismutation coefficient (Pyr. utilized in O_2)/(Pyr. utilized in N_2) will indicate the relative importance of these processes (33).

TABLE III

Utilization of Pyruvate by Potato Slices

Buffer, Ringer-phosphate, 0.01 M; pH 6.0; 3 ml.; lithium pyruvate, 0.01 M; temperature 28°C. Figures give cu. mm./mg. dry weight/hr.

| Exptl. conditions | Control cu. mm. | Pyruvate cu. mm. |
|--------------------------------------|--------------------|---------------------|
| O_2 uptake | 0.47 | 0.50 |
| CO_2 output in N_2 | 0.30 | 0.40 |
| Pyruvate utilization | | |
| In O_2 | | 0.35 |
| In N_2 | | 0.10 |

Potato slices were incubated with pyruvate in phosphate buffer with O_2 or with N_2 as the gas phase. Neither in O_2 nor in N_2 did pyruvate increase appreciably the O_2 uptake or CO_2 production. Pyruvate was utilized, however, as shown by chemical analysis, the utilization being three times greater in the presence of oxygen than in its absence. The oxidismutation coefficient was 3 (Table III). Such a discrepancy between manometric determination and chemical analysis in experiments with tissues has often been found by those working with animal tissues.

b) *Acetate*. Whether pyruvate is directly oxidized or is first decarboxylated, acetate seems to be the end product. Addition of acetate increased the O_2 uptake, sometimes slightly, sometimes, as in the experiments of Fig. 5, by 68%. Fluoroacetate, an inhibitor of acetate oxidation (34), inhibited the respiration of potato slices by 39%. Further evidence of acetate oxidation was obtained with C^{14} -carboxyl-labeled

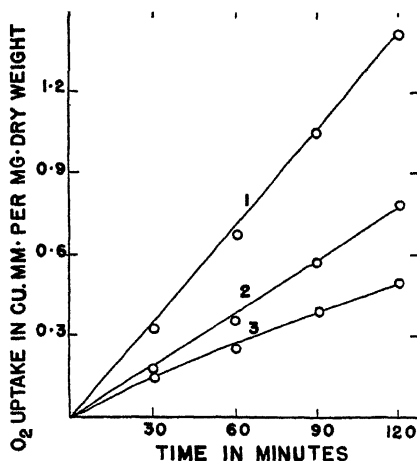


FIG. 5. Effect of acetate and fluoroacetate on the O_2 uptake of potato slices. Volume 3 ml. 1. Acetate; 2. control; 3. fluoroacetate (0.01 M).

acetate. In the first of these experiments, there was no increase in the O_2 uptake although there was utilization of acetate and appearance of $C^{14}O_2$ in the respiratory CO_2 trapped in the NaOH used in the center cup of the Warburg vessel. In the second and third experiments there was an increase in O_2 uptake. The appearance of $C^{14}O_2$ in the respiratory CO_2 is evidence of oxidation of acetate by potato slices (Table IV).

The Synthesis of Citric Acid

The metabolism of acetate in animal tissues seems to start by a condensation process between a phosphorylated acetate and oxalacetate

TABLE IV

Metabolism of Acetate by Potato Slices

Buffer, Ringer-phosphate, pH 6.0, 3.7 ml.; $CH_3C^{14}OONa$, 0.1 M , 0.4 ml. (specific activity 130,000 counts/min.). Temperature 28°C.; time 80 min.

| Dry tissue weight | O_2 uptake | Acetate utilization | $C^{14}O_2$ |
|-------------------|--------------|---------------------|-------------|
| mg. | cu. mm. | μ moles | counts/min. |
| I 206.6 | 181 | 14.5 | 14,990 |
| II 178.2 | 158 | 12.7 | 13,570 |
| III 155.0 | 129 | 11.2 | 12,900 |

to give citric acid (35,36). Aconitase transforms citric acid into isocitric acid, and the oxidation process begins through the tricarboxylic acid cycle of Krebs. In yeast, acetate seems to form both citric acid and succinic acid (37). Ingold (38) reported very low values of citric, malic, and oxalic acids in potato juice.

We have found, however, that potato tuber is rich in citric acid. The tuber was cut into small pieces and was ground in a mortar with the addition of sand. The protein of the potato juice was precipitated with an equal volume of 10% CCl_3COOH , and citric acid was determined in the filtrate. There was 0.11 mg. citric acid/ml. of original juice. Since the fluid content of the potatoes used in these experiments was 85%, a 100-g. potato contained 9.35 mg. citric acid. Several potatoes of the same variety (McClure) that were analyzed for citric acid on different days gave amounts close to that given above ($\pm 10\%$).

The metabolism of acetate in potato tuber seems to follow the same pathway as that of animal tissues, namely, condensation with oxalacetate to give citrate. The synthesis of citric acid was obtained by following the procedure of Rudolph and Barron (36) for the synthesis of citric acid in kidney suspensions, namely incubation in the presence of malonic acid to break continuity of the cycle. Forty grams of peeled potato slices was ground in a Waring Blendor with 40 ml. of half-strength Krebs-Ringer solution containing 0.01 *M* phosphate, pH 6.0. The suspension was filtered through cloth. To 6 ml. of this fluid were added 1 ml. of 0.05 *M* acetate, 0.05 *M* oxalacetate, and 0.5 *M* malonate, and 2 mg. ATP. Other flasks were prepared without acetate or without oxalacetate. They were incubated at 28°. An aliquot was taken immediately for the determination of initial citric acid, and subsequent samples (1 ml.) were taken 30 min. and 90 min. later. Trichloroacetic acid (1 ml. of 10%) was added to the aliquots and the volume was brought to 5 ml. The analysis was performed in 1 ml. of the filtrate (Table V). Synthesis of citric acid was obtained only in the flasks containing both acetate and oxalacetate. This is the first time that such a synthesis has been found in plant tissues.

Aconitase in Potato. Oxidation of citric acid starts with its transformation into isocitric acid by aconitase. This enzyme was found in potato tuber. Potatoes (600 g.) were cut into small pieces and ground with the aid of sand in a mortar. Grinding was continued for 2 hr. after addition of 150 ml. of 0.154 *M* bicarbonate. The fluid was pressed through cloth and then centrifuged. The supernatant fluid was treated with $(\text{NH}_4)_2$ -

TABLE V

Synthesis of Citric Acid by Potato Juice

Experimental details given in the text. The values of citric acid are those obtained in the 1-ml. aliquots.

| Additions | Citric acid | | |
|----------------------------------|-----------------|-----------------|-----------------|
| | Initial | 30 min. | 30 min. |
| None | $\mu g.$ 243 | $\mu g.$ 243 | $\mu g.$ 243 |
| Oxalacetate+malonate+ATP | 243 | 243 | 243 |
| Acetate+malonate+ATP | 243 | 243 | 243 |
| Acetate+oxalacetate+malonate+ATP | 243 | 282 | 300 |

SO₄ (70 g./100 ml.), and was left for 2 hr. at 3° to complete the protein precipitation. After centrifugation the precipitate was dissolved in water and dialyzed overnight at 3° with running distilled water in a rocking dialyzer. Further precipitation of the protein was effected with 5 vol. of acetone cooled to -20°. The precipitate was filtered through a Büchner funnel and was dried by suction. The protein was dissolved in 20 ml. of 0.03 *M* phosphate buffer, pH 7.4, and was incubated for 10 min. at 38°. At this time 2 ml. of 0.1 *M* *cis*-aconitate (neutralized with NaHCO₃) was added. Aliquots (4 ml.) were taken at different intervals and were added to flasks containing 4 ml. of 10% CCl₃COOH. There was a steady rate of citric acid formation (Fig. 6).

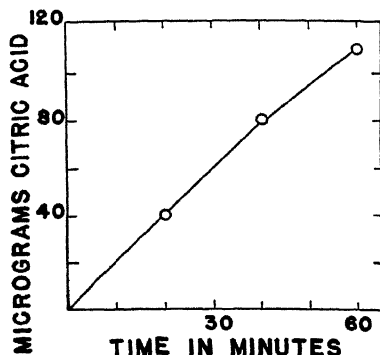


FIG. 6. The activity of aconitase from potato. The protein was dissolved in phosphate buffer, 0.03 *M*, pH 7.4; volume 3 ml.; incubation at 38°C.

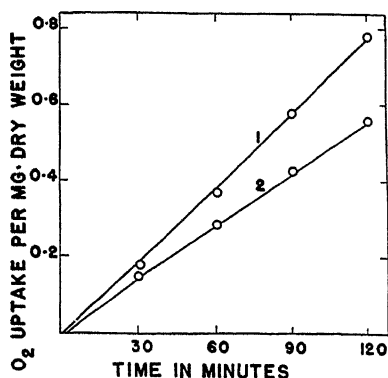


FIG. 7. Effect of malonate on the respiration of potato slices. Volume 3 ml.
1. Control; 2. malonate (0.03 M).

Oxidation of Acids of the Tricarboxylic Acid Cycle. Addition of citrate to potato slices increased the O₂ uptake by 14%. Citrate utilization was found on chemical analysis. Although the O₂ uptake of potato slices was not increased on addition of α -ketoglutarate, its utilization was demonstrated by chemical analysis. Addition of succinate, both at pH 6 (phosphate buffer) and at pH 3.8 (hippurate buffer) increased the O₂ uptake 45% and 57%, respectively (Table VI). Further evidence of the participation of succinate in the respiration of potato slices was found in the inhibition produced by malonate (Fig. 7).

Amino Acid Oxidation. Added amino acids (L-glutamic acid, L-aspartic acid, DL-alanine) had very little effect on the O₂ uptake; further-

TABLE VI

Oxidation of Acids of the Tricarboxylic Acid Cycle by Potato Slices

Figures give cu. mm./mg. dry weight/hr.

| Substrate | pH | O ₂ uptake | Utilization |
|-------------------------|-----|-----------------------|-------------|
| | | cu. mm. | cu. mm. |
| None | 6.0 | 0.38 | 0.25 |
| Citrate | 6.0 | 0.44 | |
| Succinate | 6.0 | 0.55 | |
| None | 3.8 | 0.29 | 0.4 |
| Succinate | 3.8 | 0.46 | |
| None | 6.0 | 0.39 | |
| α -Ketoglutarate | 6.0 | 0.40 | |

more, there was no extra NH_3 formation (Table VII). These experiments were performed with fresh potato slices as well as with slices kept in running tap water for 24 hr. It would seem that potato tissues do not possess amino acid oxidases.

Transamination. Transamination reactions, found in a large number of plants, have been reported by Leonard and Burris (39) in the leaves, stem, and roots of potato. For the study of transamination in potato tuber, potato slices were incubated for 3 hr. at 38° in the presence of glutamate and oxalacetate, and of aspartate and α -ketoglutarate. The transamination reaction, glutamate + oxalacetate $\xrightleftharpoons[b]{a}$ aspartate + α -ketoglutarate, was thus tested in both directions, in *a* by the deter-

TABLE VII
Effect of Amino Acids on the O_2 Uptake of Potato Slices
Buffer, Ringer-phosphate, pH 6.0; temperature 28°C . Figures
give cu. mm./mg. dry weight/2 hr.

| Kind of slices | Substrate | O_2 uptake | NH_3 |
|--------------------------|-------------|---------------------|---------------|
| | | cu. mm. | cu. mm. |
| Fresh slices | None | 0.70 | 0.12 |
| | L-Glutamate | 0.74 | 0.14 |
| | L-Aspartate | 0.74 | 0.15 |
| Slices washed for 24 hr. | None | 1.01 | 0.19 |
| | L-Glutamate | 1.00 | 0.20 |
| | DL-Alanine | 0.99 | 0.22 |

mination of glutamate utilized and aspartate formed; and in *b* by the determination of aspartate utilized and glutamate formed. In both directions the reaction was found to have reached equilibrium at the end of the incubation period (Table VIII). To test the transamination reaction, glutamate + pyruvate \rightleftharpoons α -ketoglutarate + alanine, potato slices were incubated with glutamate and pyruvate. There was no alanine formation. From these experiments it must be concluded that potato tuber contains the glutamate-ketodicarboxylic acid transaminase, but not the glutamate-ketomonocarboxylic acid transaminase.

Butyrate Oxidation. These experiments were performed with C^{14} -carboxyl-labeled butyrate. Notwithstanding the inhibition of respiration produced by butyrate, there was oxidation of this fatty acid, as shown

TABLE VIII

Transamination in Potato Slices

Buffer, 0.01 *M* phosphate pH 7.7, 5 ml.; glutamic acid, aspartic acid, alanine, pyruvic acid, α -ketoglutaric acid, 0.01 *M*; weight of potato slices (fresh) 2 g.; incubation time, 3 hr.; temperature 38°.

Reactions:

Glutamic acid + oxalacetic acid \rightleftharpoons aspartic acid + α -ketoglutaric acid. (1)

Aspartic acid + α -ketoglutaric acid \rightleftharpoons glutamic acid + oxalacetic acid. (2)

Glutamic acid + pyruvic acid \rightleftharpoons alanine + α -ketoglutaric acid. (3)

| Reaction | Substance analyzed | Initial | Final | Transamination |
|----------|--------------------|----------------|----------------|-----------------|
| | | <i>cu. mm.</i> | <i>cu. mm.</i> | <i>per cent</i> |
| I | Glutamic acid | 1120 | 495 | 55 |
| | Aspartic acid | — | 640 | 57 |
| II | Aspartic acid | 1120 | 320 | 18 |
| | Glutamic acid | — | 237 | 21 |
| III | Glutamic acid | 1120 | 1140 | None |
| | Alanine | — | None | None |

by chemical analysis and by the presence of $^{14}\text{O}_2$ in the respiratory CO_2 (Table IX).

The Terminal Steps of Respiration. The abundance of polyphenoloxidase in potato, and the discovery by Kubowitz (40) that this enzyme readily produces a number of coupled oxidation-reduction reactions led Boswell (3) to postulate that polyphenoloxidase was the main pathway for transfer of electrons from foodstuff to molecular oxygen. This contention has most recently been challenged by Levy *et al.* (13), who demonstrated that the respiration of potato slices was inhibited by CO and

TABLE IX

Metabolism of Butyrate by Potato Slices

Ringer solution containing 0.01 *M* phosphate pH 6.0, 5 ml.; butyrate containing $\text{C}_4\text{H}_7\text{CH}_2\text{CH}_2^{14}\text{OONa}$, 5 mmoles; dry weight of potato slices, 110 mg.; duration of experiment, 3 hr.; temperature 28°.

| Exptl. conditions | Volume <i>cu. mm.</i> | ^{14}C counts/min. |
|--|--------------------------|--------------------------------|
| O ₂ uptake, control | 242 | |
| O ₂ uptake, butyrate | 216 | |
| Butyrate utilization | 30 | |
| Initial butyrate | | 52,600 |
| Final butyrate | | 46,700 |
| Respiratory CO ₂ (butyrate) | 173 | 1,400 |

that the inhibition was reversed by light. Furthermore, they found cytochrome oxidase, although they were unsuccessful in their attempts to isolate cytochrome *c*.

To determine the role of copper-containing oxidases in the respiration of potato slices, 8-hydroxyquinoline was used as an inhibitor of these enzymes. Hydroxyquinoline at a concentration of 0.001 *M* inhibited completely crude polyphenoloxidase (as obtained from potato juice) at the end of 30 min. (Fig. 8). The reason for this induction period is not known. The presence of cytochrome oxidase in potato was studied with hydroquinone and ascorbic acid as the reductants of cytochrome *c*. The

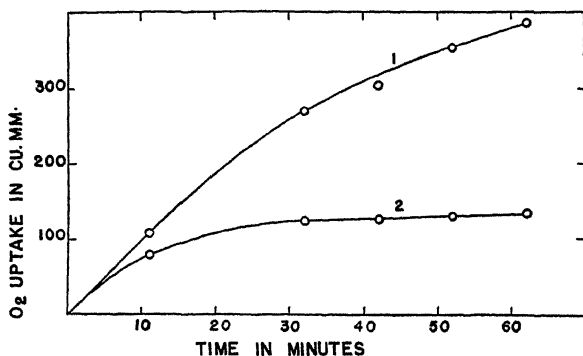


Fig. 8. Inhibition of polyphenoloxidase (potato) by 8-hydroxyquinoline, 0.001 *M*. Buffer, phosphate, 0.01 *M*, pH 7.0; catechol, 0.01 *M*; temperature, 28°C.; volume 3.0 ml. The enzyme was a ground potato suspension. 1. Control, potato suspension + catechol; 2. potato suspension + catechol + 8-hydroxyquinoline.

tissue was ground in a Waring Blendor in the presence of 5 vol. of ice-cold water. The starch was allowed to settle and was discarded. The suspension was passed through cheesecloth and was centrifuged for 30 min. at 5000 r.p.m. The supernatant fluid was discarded and the precipitate was suspended in 0.03 *M* phosphate buffer pH 7.0. Ascorbic acid and hydroquinone (0.01 *M*) were kept in the side arm of the Warburg vessels. Cytochrome *c*, 3 mg./vessel, was added to the main portion. There was a vigorous O₂ uptake in the presence of the complete system: cytochrome oxidase + cytochrome *c* + hydroquinone or ascorbic acid. In the absence of cytochrome *c* there was also some O₂ uptake, catalyzed presumably by the copper-containing enzymes present in potato tissue.

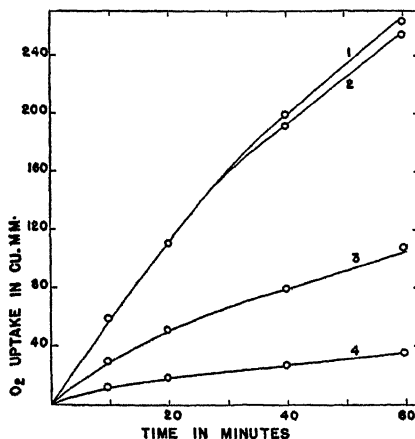


FIG. 9. Effect of 8-hydroxyquinoline on the oxidation of hydroquinone by cytochrome oxidase and by polyphenoloxidase from potato. Buffer, phosphate, 0.03 *M*, pH 7.0; volume, 3.0 ml. 1. Potato suspension + cytochrome c + hydroquinone; 2. potato suspension + cytochrome c + hydroquinone + 8-hydroxyquinoline; 3. potato suspension + hydroquinone; 4. potato suspension + hydroquinone + 8-hydroxyquinoline.

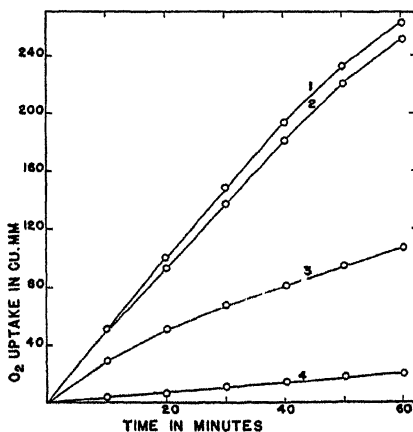


FIG. 10. Effect of 8-hydroxyquinoline on the oxidation of ascorbic acid by cytochrome oxidase and ascorbic acid oxidase (?) of potato. Buffer, phosphate 0.03 *M*, pH 7.0; hydroquinone 0.01 *M*; 8-hydroxyquinoline 0.001 *M*; volume 3.0 ml. 1. Ascorbic acid + 8-hydroxyquinoline + cytochrome c; 2. ascorbic acid + cytochrome c; 3. ascorbic acid; 4. ascorbic acid + 8-hydroxyquinoline.

The oxidation of hydroquinone and of ascorbic acid in the absence of cytochrome *c* was inhibited by 8-hydroxyquinoline (0.001 *M*), whereas, in the presence of cytochrome *c* it was not affected at all (Figs. 9 and 10).

Since 8-hydroxyquinoline inhibited the copper enzymes and had no action on cytochrome oxidase, it was used as an indicator of the role of polyphenoloxidase in the respiration of potato. It produced a partial inhibition of respiration, which had not appeared at the end of 1 hr. but reached 27% after 240 min. (Fig. 11). This long induction period may be due to the slow penetration of the inhibitor through the cell membrane. Evidence of this contention was found in the immediate inhibition observed when the slices were minced in a Latapie mincer.

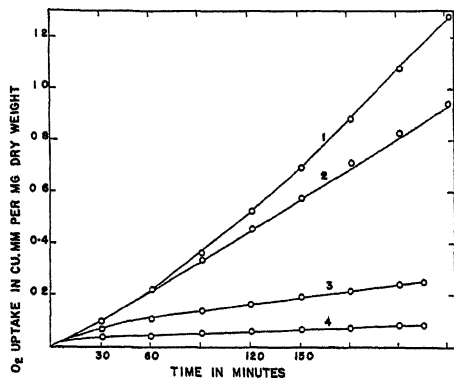


Fig. 11. Effect of 8-hydroxyquinoline on the oxygen uptake of potato slices and on ground potato. Buffer, phosphate, 8-hydroxyquinoline, 0.001 *M*; volume, 3.0 ml. 1. Slices, control; 2. slices + 8-hydroxyquinoline, 3. ground potato, control; 4. ground potato + 8-hydroxyquinoline.

Borate (0.01 *M*) had no effect on the respiration of potato slices; diethyldithiocarbamate (0.01 *M*) inhibited it 20%. Both reagents have been used as inhibitors of reactions catalyzed by polyphenoloxidase.

The presence of cytochrome *c* in potato tissues was reported by Keilin (41). Two attempts were made to isolate cytochrome *c*, the first using Keilin and Hartree's method (42), the second Goddard's modification (43). Melanine formation was diminished by addition of 8-hydroxyquinoline. No cytochrome *c* was detected.

DISCUSSION

The experiments presented in this paper, together with previous investigations on the metabolism of carbohydrates by potato tuber, are an indication that, in its main features, the pathway is the same as that found for yeast and for animal tissues. The phosphorylation process seems to start the metabolic reactions, since starch phosphorylase is present in the tissue. The anaerobic degradation to pyruvic acid must follow the series of reactions of the Embden-Meyerhof scheme. In fact, the presence of zymohexase has been shown. An interesting finding is that pyruvic acid in its anaerobic metabolism utilized the two pathways: alcohol and lactic acid fermentation. The presence of carboxylase was demonstrated, and the reduction of acetaldehyde to alcohol was shown by the analysis of alcohol formed on fermentation. The utilization of the second pathway was demonstrated by the isolation of lactic dehydrogenase and by analysis of the lactic acid formed. The reduced diphosphopyridine nucleotide (DPNH_2) formed on oxidation of glycerophosphoaldehyde is thus subject to the competing action of acetaldehyde reductase and lactic dehydrogenase. Warburg (44) has stressed the great affinity of DPNH_2 for the activating protein of acetaldehyde reductase (alcohol dehydrogenase). The ratio of alcohol:lactic acid formation, 17:1, was considerably in favor of alcohol fermentation.

The aerobic phase of carbohydrate metabolism starts with the oxidation of pyruvate. Its greater utilization in the presence of O_2 is an indication of this oxidative pathway. Acetate, the probable product of this oxidation, was oxidized by potato slices, as shown by the presence of C^{14}O_2 in the respiratory CO_2 when $\text{CH}_3\text{C}^{14}\text{OONa}$ was incubated with potato slices. The condensation of acetate with oxalacetate to form citrate was demonstrated. Since similar findings have been observed in tomato stems³ it seems that this condensation process may be found in many plants. Furthermore, aconitase was found in potatoes, and the oxidation and utilization of the acids belonging to the tricarboxylic acid cycle (citrate, α -ketoglutarate, succinate) were demonstrated. The presence of these processes favors the view that carbohydrate oxidation in potato occurs through the tricarboxylic acid cycle of Krebs.

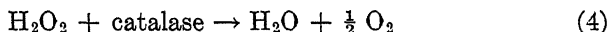
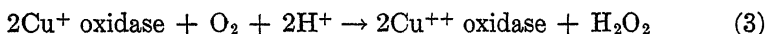
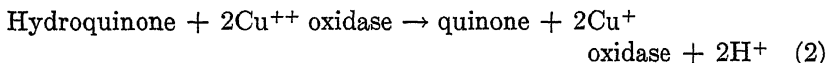
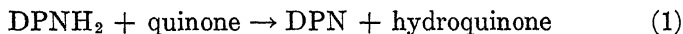
The oxidation of butyric acid, as shown by its utilization and respiratory CO_2 formation, is an indication that the potato tuber contains the necessary enzyme systems for the oxidation of fatty acids. Concerning

³Unpublished observations.

nitrogen metabolism, the lack of amino acid oxidases and the presence of transaminases speaks in favor of the utilization of this last reaction for the performance of those processes concerned with the formation of proteins.

In experiments with 8-hydroxyquinoline it has been shown that while polyphenoloxidase was inhibited almost completely after an induction period of 30 min., cytochrome oxidase was not affected at all. With this reagent it was found that partial inhibition of the respiration of potato slices started after an induction time of 1 hr. This partial inhibition of respiration is an indication that potato tissue might utilize both oxidative pathways of electron transfer: cytochrome oxidase and polyphenol-oxidase.

It is difficult to establish definitely the *actual* share of the two possible final steps of electron transfer to molecular oxygen: polyphenol-oxidase and cytochrome oxidase. Warburg (45) states that in the presence of polyphenoloxidase there is a coupled oxidation-reduction between reduced pyridine nucleotides (di- and tri-) and polyphenols:



Such reactions make possible the transfer of electrons from the following components of the Krebs cycle: isocitric acid [triphosphopyridine nucleotide (TPN)], malic acid (DPN and TPN), and α -ketoglutaric acid (DPN). There may also be transfer from lactic acid (DPN) and from alcohol (DPN). There can be no transfer, however, on the oxidation of succinic acid to fumaric acid, for this oxidation is not catalyzed by pyridine nucleotide: it must go through the cytochrome system or directly from flavoprotein to molecular oxygen.

SUMMARY

The respiration of freshly cut potato slices, although lower than that of long-washed slices, gives a respiratory quotient of 1, an indication of complete oxidation of carbohydrate.

8-Hydroxyquinoline, after an induction period, inhibited completely

polyphenoloxidase from potato, while it had no effect on cytochrome oxidase. The respiration of potato slices was only partially inhibited. This is an indication that both systems take part in respiration.

Carbohydrate fermentation in potato seems to follow the same pathway as in yeast and in animal tissues. Zymohexase, carboxylase, and lactic dehydrogenase were found in this tissue. The end products of fermentation were alcohol and lactic acid, which were found in a ratio of 17:1.

The oxidative pathway of carbohydrate oxidation seems to be accomplished through Krebs's tricarboxylic acid cycle. Synthesis of citric acid occurred on addition of acetate, oxalacetate, and adenosine triphosphate. Aconitase was found in potato. Citrate, α -ketoglutarate, succinate, pyruvate, and acetate were oxidized. With the aid of C^{14} -carboxyl-labeled acetate, $C^{14}O_2$ was found in the respiratory CO_2 . Butyrate was oxidized by potato slices, as demonstrated by the presence of $C^{14}O_2$ on incubation with $CH_3CH_2CH_2C^{14}OONa$. Transamination of glutamic and aspartic acids in the presence of oxalacetic and α -ketoglutaric acids took place rapidly while transamination between glutamic and pyruvic acid did not occur. It seems that potato tuber contains no amino acid oxidases.

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Immunological Studies On Egg White Proteins.

III. Quantitative Immunochemical Studies Of Ovomucoid ¹

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INTRODUCTION

A marked interest in ovomucoid has been stimulated by the finding that the antitryptic activity of chicken egg white is associated with this protein (1). This has been followed by a study of the amino acid residues essential for this property (2). In addition, ovomucoid prepared by less drastic conditions than those usually employed has been characterized by physicochemical methods (3). It appeared desirable to correlate the latter studies with an immunochemical investigation of this protein. The quantitative precipitin method allows for an assay of the amount of ovomucoid in chicken egg white. The immunological reactions of this protein and certain of its derivatives with rabbit anti-ovomucoid serum have also been studied quantitatively.

MATERIALS AND METHODS

Ovomucoid

The protein was prepared from fresh egg white by using a combination of sodium trichloroacetate precipitation with ethanol fractionation (3). A single sample was further reprecipitated four times to ensure the absence of ovalbumin and was used throughout the study.

Preparation of Rabbit Antisera

The first course of immunization consisted of three intraperitoneal injections of 25 mg. of ovomucoid absorbed on alumina administered at 5-day intervals. This was

¹ This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

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followed by a course of five subcutaneous injections of 25 mg. of protein suspended in paraffin oil at intervals of 6 days. These procedures failed to effect any significant antibody production. The technique of Freund and Bonanto (4) was then employed for a third course of immunization. Two 15-mg. portions of ovomucoid were administered subcutaneously at 14-day intervals. An anti-serum of relatively low titer was thus obtained.

Preparation of Ovomucoid Derivatives

Iodinated, acetylated, coupled, and methylated ovomucoids were prepared by the methods of Fraenkel-Conrat *et al.* (2). At the conclusion of the reactions, the mixtures were dialyzed against running tap water (approximately 5°C.) for 18 hr. and against cold distilled water for an additional 24 hr. The derivatives were lyophilized and for immunological studies they were dissolved in saline-borate buffer (pH 7.3).

Quantitative Precipitin Reactions

Total nitrogen was determined on exhaustively dialyzed solutions of ovomucoid. The quantitative precipitin reactions and tests of the supernatant solutions to the specific precipitates for excess antigen and antibody were carried out as previously described (5). The antigen-antibody mixtures were stored at 1-2°C. for 5 days prior to the removal of the specific precipitates.

RESULTS

A plot of the data for the reaction of ovomucoid with anti-ovomucoid rabbit serum gives the typical rabbit-type curve shown in Fig. 1. There is evidence of significant "tailing off" in the extreme antigen excess region, which indicated that the antigen employed contains impurities. The agar diffusion technique of Oudin (6,7) demonstrates that there are at least two components present in this preparation of ovomucoid.³

Tests of the supernatant solutions of the precipitin reaction revealed a very narrow antibody excess region with no well-defined equivalence zone. The approximate position of the latter is designated by an arrow in Fig. 1. The ratio of (antibody N)/(antigen N) at the equivalence point as calculated from the Heidelberger-Kendall equation (8) is 14.6. This value is somewhat higher than that reported by Hooker and Boyd (9) for ovalbumin. This is to be expected since ovomucoid has a lower molecular weight.

It is possible to assay egg white for ovomucoid by reacting it with the ovomucoid antiserum. The results of this precipitin reaction are plotted in Fig. 1. It will be observed that the agreement of the curves plotting the results of the reactions of ovomucoid and egg white are

³ These experiments were carried out by Dr. E. L. Becker.

extremely good over the entire range studied, even in the antigen excess region. A comparison of the ratio of ovomucoid N to egg white N which gives the same amount of specific precipitate in the region of antibody excess makes it possible to estimate the ovomucoid content of egg white. A value of 9.3% of the total egg white N is obtained. This may be expressed in terms of protein content if it is assumed that egg white contains 16% and ovomucoid 13.2% nitrogen. By employing this correction it was found that 11.3% of the protein in egg white is ovomucoid. This is in close agreement with electrophoretic (10) and other assay values (1,11).

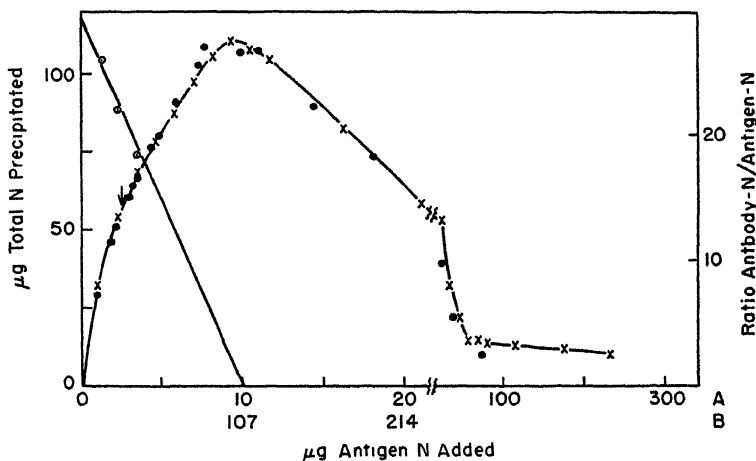


Fig. 1. The reaction of rabbit anti-ovomucoid serum with ovomucoid —x—x—x— (A abscissa values) and with chicken egg white —●—●—●— (B abscissa values).

The immunochemical reaction of the modified ovomucoids with rabbit anti-ovomucoid serum differ markedly. The iodinated protein appears to be affected very little, while the acetylated, coupled, and methylated samples, respectively, display decreasing specificity. The extent of cross reaction was estimated by comparing the maximum amount of antibody specifically precipitated in each case. More precise estimations are not possible because of failure of these protein derivatives to show immunochemical homogeneity. Considerations of possible molecular-weight variations, changes in nitrogen content, and denaturation effects are other factors necessitating such an estimation. The immunological

results are summarized in Table I along with data of Fraenkel-Conrat *et al.* (2) on the extent of the chemical modification effected. Since the ovomucoid derivatives were prepared under the same conditions as outlined by the above workers, it is assumed that the product obtained possessed very nearly the properties reported by these investigators. The amount of purified ovomucoid available for preparing derivatives was insufficient to allow for characterization of the latter.

Iodination of ovomucoid reduced the amount of nitrogen precipitated at the maximum by approximately 10%. The fact that only 6% of the tyrosine residues have probably been substituted suggests that these groups are involved in the formation of the specific precipitate. Acetyla-

TABLE I
*Effect of Various Chemical Modifications of the Ovomucoid Molecule
on Its Immunological Specificity*

| Nature of reaction | Type ^a | Approx. portion of groups reacted ^a | Approx. reduction in maximum total N pptd. |
|--------------------|----------------------|---|--|
| | | <i>per cent</i> | <i>per cent</i> |
| Acetylation | Amino | 77 | 35 |
| Esterification | Carboxyl | 60 | 75 |
| Iodination | Primarily phenol | 6 | 10 |
| Coupling | Imidazole and phenol | 100 | 50 |

^a Taken from data of Fraenkel-Conrat *et al.* (2).

tion of a protein with acetic anhydride has been found to be specific for amino groups. Treatment with this reagent, which results in the acetylation of approximately 75% of the amino groups, decreased the quantity of nitrogen precipitated at the maximum by about 35%. Thus it would appear that not all of the amino groups are involved in the reaction of ovomucoid and its antibody. The necessity of free carboxyl groups is demonstrated by the reactions of methylated ovomucoid. A reduction of 75% was obtained in the nitrogen precipitated at the maximum when this derivative was reacted with the rabbit anti-ovomucoid serum. Diazobenzenesulfonic acid, under the conditions used in this study, couples with approximately all of the imidazole and phenol groups present in ovomucoid. This treatment results in a reduction of approximately 50% of the nitrogen precipitated at the maximum.

DISCUSSION

The results of the quantitative precipitin reaction indicate that ovomucoid, as currently prepared, is a heterogeneous protein. Further support for this is seen by the results of the Oudin technique (6,7) which demonstrates the presence of at least two antigenic components. Physical data (3), especially electrophoresis experiments in 0.01 ionic strength buffers, indicate extreme heterogeneity. However, the quantitative precipitin curve does not appear to be nearly as complex as might be expected.

The precipitin reaction serves as a satisfactory assay tool, especially in complex mixtures. The value of 11.3% for the ovomucoid content of chicken egg white agrees quite well with those observed in the literature. Lineweaver and Murray (1) found that about 12% of chicken egg white proteins could be accounted for in terms of trypsin inhibitor activity. Electrophoretic analysis as carried out by Longsworth *et al.* (10) suggest that ovomucoid constitutes approximately 14% of this system. A value of 10% was reported by Meyer (11) for an isolation procedure.

It is known that the immunological specificity of proteins is determined by certain chemical groupings in the molecule and that this can be altered by the introduction of definite chemical "determinant" groups. Heidelberger and co-workers (12,13) observed that the immunological specificity of ovalbumin was markedly altered by the introduction of chemical groups. The present investigation shows that the specificity of purified ovomucoid is changed by acetylation, esterification, and coupling.

A notable feature of the immunochemical reactions of the ovomucoid derivatives is the increased precipitation in the antigen excess region. Similar observations have been reported by Heidelberger and his group (12,13) and Kleczkowski (14). The latter suggested that chemical treatment may alter the physical properties of the antigen with a resultant reduction in the solubility of complexes in the region where the unmodified antigen exhibits a soluble complex with its antibody. In this study it was noted that the methylated protein exhibits complete abolition of such a zone over the range studied, although the extent of maximum nitrogen precipitated was greatly diminished.

SUMMARY

Ovomucoid, as currently prepared, has been found to be immunologically nonhomogeneous by both the quantitative precipitin reaction

and the Oudin technique. Rabbit antisera to ovomucoid, however, can be used to assay egg white for this protein. Studies of the reactions of chemically modified ovomucoid with antiserum to the parent protein have been carried out.

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Certain Chemical Characteristics of Irradiated Pyridoxamine with Reference to Antibacterial Properties¹

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INTRODUCTION

In the preceding communication (1) it was reported that an antibacterial agent was obtained by ultraviolet irradiation of acidic aqueous solutions of pyridoxamine. In the course of the present investigation it has been observed that the irradiation products, in contradistinction to pure pyridoxamine and pyridoxine, exhibit reducing properties and release ammonia on treatment with an excess of strong alkali. In order to study the significance of these reactions, quantitative analytical methods were applied and the results correlated with the spectral characteristics, the concentration of the photochemically transformed material, and antibacterial potency.

EXPERIMENTAL

Light Source

An ultraviolet quartz burner (General Electric, Uviarc AH-1) was placed vertically over the agitated solution at a distance of 50 cm. from its surface and irradiated through a Corning light filter No. 9863.

Assay of Antibacterial Activity

The previously described method (2) was employed. Antibacterial potency was expressed in units/mg., a unit being equal to the reciprocal of the minimal concentration (in mg./ml.) causing complete inhibition of growth of *Escherichia coli*, strain 42, after incubation at 37° for 24 hr.

For irradiated solutions, the potency as well as other quantitative results including the extinction coefficients were calculated on the basis of the concentration of the compounds (hydrochlorides of pyridoxamine and pyridoxine) before irradiation.

¹ This investigation was made possible by grants from the Commonwealth Fund and the National Vitamin Foundation.

Irradiation of Pyridoxamine

Present experiments on irradiations of larger volumes (100 ml.) of aqueous pyridoxamine dihydrochloride solutions adjusted to pH 2 with hydrochloric acid confirmed the previous observations (1), except that lower potencies were obtained, obviously due to the changed ratio of irradiated surface to the volume of the solution. Decreasing concentrations of pyridoxamine yielded increasing potencies and required shorter optimal times of irradiation. The concentration (5 mg./ml.) chosen for this investigation represented, necessarily, a compromise between the desired objectives of obtaining a maximum of potency as well as high concentrations of the antibacterial product. The introduction of 95% ethanol, acidified so that the resulting solution was 0.004 *N* with respect to hydrochloric acid, as solvent increased the potency two- to threefold when compared with corresponding aqueous solutions (Nos. 2 and 9, Table I). Furthermore, the activated ethanolic solutions could be concentrated to a small volume under reduced pressure, in an inert atmosphere and at temperatures not exceeding 35° without appreciable loss of activity. Concentrates containing up to 1400 units/mg. were thus obtained. The rate of thermal inactivation of irradiated ethanolic solutions and their concentrates was slow up to 40° but increased rapidly at higher temperatures.

Solid Products by Precipitation with Acetone

Attempts to obtain active products in a solid state from irradiated pyridoxamine solutions by removal of the solvent failed because of almost complete inactivation. A series of solid active fractions could be prepared by adding acetone to the concentrates derived from ethanolic solutions until an incipient, permanent turbidity was formed; on standing at 5° and frequent stirring a crystalline precipitate separated. This procedure was applied to the filtrate and to each consecutive one until no turbidity occurred. Further precipitates could be obtained by concentrating the final filtrate at 35° bath temperature, under reduced pressure and in a current of nitrogen to a small volume, and addition of acetone. From ethanolic concentrates of relatively low potency (80–150 units/mg.), the first precipitate obtained was inactive and consisted mostly of unreacted pyridoxamine dihydrochloride corresponding to 40–50% of the initial concentration. The fractions obtained thereafter showed a varying degree of activity, the most active one appearing

usually as the second precipitate; its potency represented an increase of 70%. All precipitates obtained from more potent material (250–350 units/mg.) showed antibacterial activity corresponding to a 40–50% recovery. The most active fractions exhibited, on the average, a two-fold increase in potency. The precipitates, tan to brown crystalline substances, represented mixtures of pyridoxamine with its irradiation products. Their melting points ranged from 180–200° (decomposition). The total nitrogen (Kjeldahl) was lower than that of pyridoxamine dihydrochloride and varied from 9.5 to 10.9% (Table II).

The thermostability of the fractional precipitates appeared considerably increased. When dried over phosphorus pentoxide and subsequently heated for 1-hr. periods while protected against atmospheric moisture, the potency remained unchanged up to 100°. Complete inactivation occurred at 150°.

Irradiation of Pyridoxine

The previous observation (1) that aqueous solutions of pyridoxine are only slightly activated was confirmed. However, irradiation of solutions in 95% ethanol acidified to 0.004 *N* with hydrochloric acid produced considerable antibacterial potency. Solutions containing 5.0, 1.0, and 0.1 mg. of pyridoxine hydrochloride/ml. were activated, on the average to the respective potencies of 150, 600, and 1600 units/mg. after irradiation periods of 29, 15, and 5 hr., respectively. The concentration of 1 mg./ml. was chosen for this investigation because it produced levels of activity comparable to those obtained from pyridoxamine.

Ultraviolet Absorption Spectra

Absorption spectra were determined in 1-cm. quartz cells in a Beckman photoelectric quartz spectrophotometer Model NU, on solutions diluted with triple-distilled water to a concentration of 20 µg./ml. and adjusted to pH 3.

Qualitative Reactions

The following reactions, not given by pyridoxamine and pyridoxine, were positive with irradiation products: Tollens' ammoniacal silver nitrate, Fehling's reagent, Nelson's copper sulfate–arsenomolybdate reagent (3); heating of acidic solutions, color change from golden-yellow to brown; excess of strong alkali and heating, dark brown coloration and evolution of ammonia. The tests for pyridoxamine with ferric chloride (4) and 2,6-dichloroquinone chloroimide (4–7) were positive with the irradiation products apparently due to the presence of unreacted material.

TABLE I—Irradiated Pyridoxamine and Pyridoxine Solutions

| Prepa. no. | Conc. (mg./ml.) | Irradiation time in hrs. | Units/mg. | Conc. of unreacted material ^a | Ammonia N | | | Apparent extinction coefficient | | |
|---|--------------------|-----------------------------|-----------|--|--------------------|-------------------|--|---------------------------------|---------------------------------|---------------|
| | | | | | Found ^b | Calcd. | Mole NH ₃ per mole reacted (6) : (7) | At 293 mμ. | Per cent of 545 ^b | At 228 mμ. |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) |
| Pyridoxamine dihydrochloride in 95% ethanol | | | | | | | | | | |
| 1 | 5 | 4 | 56 | 77.58 | 1.20 | 1.30 | 0.92 | 290 | 53 | 170 |
| 2 | 5 | 11 | 160 | 52.12 | 2.70 | 2.78 | 0.97 | 250 | 46 | 260 |
| 3 | 5 | 19 | 240 | 32.45 | 4.16 | 3.93 | 1.06 | 178 | 38 | 295 |
| 4 | 5 | 27 | 320 | 18.91 | 5.00 | 4.71 | 1.06 | 138 | 25 | 320 |
| 5 | 5 | 35 | 300 | 11.75 | 5.60 | 5.13 | 1.09 | 102 | 19 | 310 |
| 6 | 5 | 29 ^c | 200 | 38.02 | 3.95 | 3.60 | 1.10 | 186 | 34 | 284 |
| 7 | 5 | 4 ^c | 56 | 69.10 | 1.18 | 1.80 | 0.66 | 254 | 47 | 160 |
| Pyridoxamine dihydrochloride in aqueous solutions | | | | | | | | | | |
| 8 | 5 | 10 | 80 | 62.92 | 1.69 | 2.15 | 0.78 | 275 | 50 | 186 |
| 9 | 5 | 29 | 140 | 18.85 | 3.99 | 4.72 | 0.85 | 100 | 18 | 239 |
| 10 | 10 | 29 | 120 | 33.10 | 2.93 | 3.90 | 0.75 | 174 | 32 | 262 |
| Pyridoxine hydrochloride in 95% ethanol | | | | | | | | | | |
| 11 | 1 | 4 ^c | 100 | 85.20 | 1.07 | 1.01 | 1.06 | 240 | 54 ^f | 90 |
| 12 | 1 | 11 ^c | 180 | 65.50 | 2.00 | 2.35 | 0.85 | 275 | 68 | 202 |
| 13 | 1 | 19 | 400 | 41.00 | 3.10 | 4.02 | 0.77 | 210 | 52 | 276 |
| 14 | 0.1 | 19 ^c | 1050 | 9.0 | 3.30 ^e | 2.80 ^d | 0.72 | | | |

^a Calculated on basis of the concentration before irradiation.^b $E_{1\text{cm}}^{1\%}$ value for pure pyridoxamine dihydrochloride at pH 2.^c Concentrates.^d After reabsorption on mercuric oxide.^e Solar irradiation.^f Per cent of $E_{1\text{cm}}^{1\%} = 400$ at 293 mμ of pyridoxine hydrochloride in water, pH 2.3.^g Nesslerization.

Determination of the Concentration of Unreacted Pyridoxamine and Pyridoxine by Means of the Indophenol Reaction

The modification by Hochberg, Melnick, and Oser (6) of the procedure of Scudi (7), based on the indophenol reaction with 2,6-dichloroquinone chloroimide was adapted. The persistence of the color for a period of 5 min. was achieved by increasing the pH of the ammonia-ammonium chloride buffer to 8.5. Furthermore, it was found that reproducible results were obtained by reading optical density at its maximum value rather than after a definite time interval. Parallel standards made with known concentrations of the pure compounds were used employing a Coleman Universal Spectrophotometer Model 11. The over-all deviation from the average value was 0.4%, and the largest deviation 1.4%. The possibility that the irradiation products interfered with the indophenol reaction was excluded by the analysis of samples of irradiated solutions containing admixtures of pure pyridoxamine and pyridoxine, respectively. Such solutions showed complete recovery of added amounts.

Determination of Ammonia

Quantitative liberation of ammonia from irradiated pyridoxamine and pyridoxine preparations in the presence of an excess of strong alkali was obtained by aeration at 100° or steam distillation. At room temperature, even prolonged aeration or microdiffusion [Conway and Byrne (8)] gave lower values by 40 and 60%, respectively,

TABLE II
Solid Products from Irradiated Pyridoxamine

| Prepn. no. | Units/mg. | Pyridoxamine concentration | Total N (Kjeldahl) | N from reacted portion | Ammonia N | | Amino N | | Apparent extinction coefficient | | |
|------------|-----------|----------------------------|--------------------|------------------------|-----------|----------------------------------|---------|---|---------------------------------|---|-----------|
| | | | | | Found | (6) expressed in per cent of (5) | Found | Calcd. for the reacted portion ^a | At 293 mμ | Col. (10) in per cent of $E_{1\%}^{1\text{cm}} = \frac{E}{545}$ | At 228 mμ |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) |
| | | % | % | % | % | | % | % | | | |
| 15 | 140 | 69.96 | 10.86 | 2.75 | 1.25 | 45.79 | 6.71 | 2.65 | 238 | 44 | 225 |
| 16 | 150 | 47.98 | 10.89 | 5.32 | 2.05 | 38.57 | 7.60 | 4.81 | 234 | 43 | 255 |
| 17 | 200 | 52.34 | 10.55 | 4.47 | 1.85 | 41.40 | 7.16 | 4.12 | 199 | 37 | 214 |
| 18 | 320 | 29.08 | 9.47 | 6.09 | 2.70 | 44.33 | 7.71 | 6.02 | 115 | 21 | 291 |

^a See explanation in the text.

which may indicate that the ammonia is not liberated from an ammonium salt. A satisfactory proof that the volatile base was identical with ammonia was obtained by the application of two additional methods: (a) Nesslerization was carried out on distillates collected in excess acid and freed from interfering volatile degradation products by aeration prior to the addition of the reagent. (Nos. 6 and 13, Table I); (b) yellow mercuric oxide, which absorbs ammonia but not aliphatic amines [François (9)], was used on steam distillates according to the procedure of Pugh and Quastel (10) with results showing a 95% and 98% recovery of the amounts obtained by titration (Nos. 6 and 13, Table I).

Amino Nitrogen

Van Slyke's micro method (11) employing nitrous acid and the volumetric gas apparatus was applied to aqueous solutions of pyridoxamine dihydrochloride which were kept protected from direct light. The amount of amino nitrogen obtained was equivalent to its one aminomethyl group.

Anal. $C_8H_{12}N_2O_2 \cdot 2 HCl$ (241.1). Calcd., amino N 5.81; found 5.73.

Since alcohol interfered with the determination (11), only the active solid products were investigated (Table II).

Reducing Capacity of Irradiated Pyridoxamine

The microtitration method of Miller and Van Slyke (12) was slightly modified by using 2 ml. of 0.25% alkaline ferricyanide reagent for each 1 ml. sample containing 1 mg. of solute and diluting the reaction mixture with an equal volume of water before titration with 0.005 *N* ceric sulfate solution. Pyridoxamine was found to consume about 1 equiv.

TABLE III

Relative Reducing Power of Irradiated Pyridoxamine

Irradiation time, potency and pyridoxamine content are listed in Tables II and III.

| Ethanollic solutions | | | Solid products | | |
|----------------------|---|-----------------|----------------|---|-----------------|
| Prepn. no. | Pyridoxamine equivalents per weight unit of | | Prepn. no. | Pyridoxamine equivalents per weight unit of | |
| | Solute ^a | Reacted portion | | Substance | Reacted portion |
| (1) | (2) | (3) | (1) | (2) | (3) |
| 3 | 2.65 | 2.88 | 15 | 1.53 | 2.75 |
| 4 | 2.63 | 3.01 | 16 | 1.80 | 2.52 |
| 5 | 2.65 | 2.87 | 17 | 1.76 | 2.60 |
| 6 | 1.87 | 2.42 | 18 | 2.27 | 2.79 |

^aCalculated on basis of the concentration before irradiation.

ceric sulfate/mole. The reducing capacity of the irradiation products was therefore determined by the relative increase in the consumption of the oxidant. With each series of determinations, parallel titrations of a standard pyridoxamine solution were carried out. The ratio of the volumes of the standard ceric sulfate solution consumed by equal amounts of the irradiated product and of pure pyridoxamine gave the number of pyridoxamine equivalents of reduction for equal preparation (Table III).

RESULTS AND DISCUSSION

It was reported previously (1) that in acidic aqueous solutions the extinction coefficient of the band characteristic for pyridoxamine (293 $m\mu$) decreased on irradiation while a new absorption appeared at a shorter wavelength. In irradiated ethanolic solutions as well as in the active fractional precipitates studied in the present investigation, a distinct new absorption band was observed at 225-230 $m\mu$ (Fig. 1), the intensity of which increased with the time of irradiation and with the rise of antibacterial activity (Table I). The conversion of pyridoxamine into its photochemical reaction products, indicated by the lowering of the extinction coefficient at 293 $m\mu$, was further demonstrated by the decreasing pyridoxamine concentrations as determined by the indophenol reaction (column 5, Table I) and by the isolation of an active, solid product, obtained by repeated fractional precipitation with acetone, which exhibited practically no absorption at 293 $m\mu$ and a sharp maximum at 233 $m\mu$ ($E_{1\%}^{1\text{cm}} = 384$) (Fig. 1, curve 4). It is also significant that this peak disappeared on thermal inactivation.

Absorption spectra of irradiated pyridoxine solutions were similar, except that no resolution of the new band was observed. Determinations of the pyridoxine content at different levels of antibacterial potency (Table I) suggest the same interpretation as the one given above for pyridoxamine.

The amount of ammonia liberated from irradiated solutions of pyridoxamine as well as of pyridoxine on heating with an excess of strong alkali increased with the duration of exposure to ultraviolet radiation. Each mole of photochemically reacted compound, computed from the values obtained by the indophenol method (column 5, Table I), released 0.7-1.1 moles ammonia. When pure pyridoxamine or pyridoxine was kept protected from extensive exposure to direct light, no volatile base was obtained.

The fact that ammonia is obtained from irradiated pyridoxine permits but one interpretation, namely, that of a fission of the pyridine nucleus. This is also suggested by the evaluation of the total nitrogen (Kjeldahl) and the amino nitrogen of the solid fractions obtained from

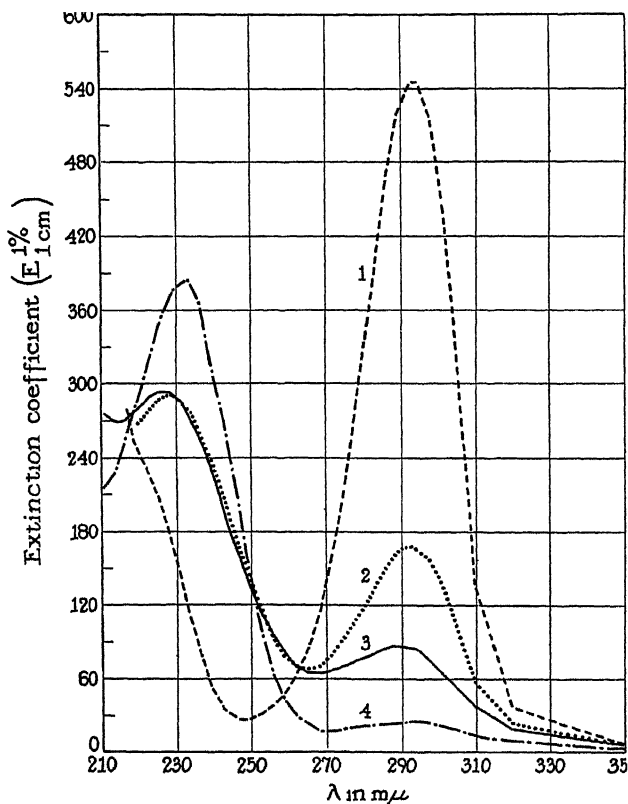


Fig. 1. Ultraviolet absorption spectra: pyridoxamine dihydrochloride (curve 1); irradiated pyridoxamine dihydrochloride in 95% ethanolic solution, 190 units/mg. (curve 2); fractional precipitates from activated pyridoxamine: 300 units/mg. (curve 3), 370 units/mg. (curve 4). Solutions diluted with water and adjusted to pH 3.

activated pyridoxamine (columns 4 and 8, Table II). When these values are corrected for the contribution made in each case by the residual, unchanged pyridoxamine (column 3, Table II, determined by the indophenol method), the values for the photochemically reacted

portion is obtained. The good agreement of the two series of values (columns 5 and 9, Table II) indicated that all nitrogen in the reacted portion appears as amino nitrogen, and that in addition to the amino group originally present in the pyridoxamine molecule a new source of amino nitrogen was formed.

The assumption of a fission of the pyridine nucleus is supported by an analogous reaction described in the literature. Freytag and Neudert (13) observed that pyridine decomposed when subjected to ultraviolet radiation. Lieben and Getreuer (14) found that ammonia was formed when irradiated pyridine was distilled with magnesium oxide, while Feigl and Anger (15) furnished evidence that the photochemical reaction product was identical with glutaconic aldehyde. The latter finding is in agreement with the reducing properties we have observed on irradiated pyridoxine and pyridoxamine. The reducing power of the photochemically reacted portion of irradiated pyridoxamine preparations in alkaline ferricyanide solutions under the experimental conditions used was found to be, on the average, 2.7 times greater than that of pure pyridoxamine (Table III).

ACKNOWLEDGMENTS

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SUMMARY

The antibacterial activity produced by the irradiation of pyridoxine and pyridoxamine was considerably increased by the use of ethanol as solvent. Concentration of activated pyridoxamine solutions followed by fractional precipitation with acetone yielded solid products of higher potency and increased thermostability.

The following properties of the irradiation products were investigated in relation to the amounts of the photochemically transformed compounds and the antibacterial potency: (a) the formation of the absorption band at 220–230 $m\mu$; (b) the release of ammonia on treatment with strong alkali; (c) the relative reducing capacity of irradiated pyridoxamine preparations; (d) the amino nitrogen in relation to the total nitrogen determined in the fractional precipitates obtained from irradiated pyridoxamine.

The results were interpreted as suggesting a fission of the pyridine nucleus in analogy to the photochemical degradation of pyridine reported in the literature.

Studies concerned with the structure of the photochemical reaction products are in progress and will be reported at a later date.

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The Inactivation of Insulin by Tissue Extracts.

VI. The Existence, Distribution and Properties of an Insulinase Inhibitor^{1, 2}

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In previous communications, we described the existence in liver extracts of a system which inactivates insulin during *in vitro* incubation. For convenience this system has been called insulinase (1). During the course of attempts to purify this system, observations were made which indicated the presence in these same extracts not only of insulinase, but also of a substance which inhibited the activity of the insulin-destroying system. The present report deals with the demonstration and some of the properties of this insulinase inhibitor.

METHODS

The method for the estimation of insulinase activity consists of observing the effect of the injection of incubated mixtures of liver extracts and insulin on the blood sugar of the fasted rabbit (1). If the insulinase activity is high, most of the insulin is destroyed during the incubation and the subsequent injection of the reaction mixture has very little hypoglycemic effect. Substances and conditions which inhibit the activity of insulinase and thereby prevent the destruction of insulin are detected by virtue of the marked hypoglycemic activity of the injected incubated reaction mixtures. The "pooled percentage" is used to express the degree of the hypoglycemic effect; the higher the "pooled percentage," the greater the insulinase activity.

All blood sugar levels cited in this report were estimated according to the Nelson modification of the Somogyi procedure (2). Other techniques that were employed are noted in the appropriate sections.

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RESULTS

The observation which directed our attention to the possible existence of an insulinase inhibitor was the increase in the insulinase activity of liver extracts following precipitation of the active material from crude extracts by acetone. Under appropriate conditions, the insulinase activity of these extracts can be precipitated almost quantitatively by the addition of acetone (1). Such precipitates, when redissolved in water and reconstituted to the same volume as that of the extracts from which they had been prepared, were invariably noted to be more active than the crude material.

The typical examples listed in Table I suggested that an inhibitor had been removed in the acetone-soluble supernatant solution. The

TABLE I
*Comparison of Insulinase Activity of Crude Beef Liver Extract
and Reconstituted Acetone Precipitates*

The acetone precipitate in *A* consisted of that fraction of the crude extract precipitated by the addition of $\frac{1}{2}$ volume of ice-cold acetone at pH 6.2. The precipitate was redissolved in water and adjusted to the same volume as that of the extract from which it had been precipitated. The crude extract in *B* consisted of an old lyophilized crude extract, similar to that in *A*, reconstituted to its original volume. The acetone precipitate in *B* was prepared by the addition of $\frac{1}{2}$ volume of acetone at pH 5.8 but otherwise corresponded to that in *A*. All incubations for 30 min. at 37°C. and pH 8.0.

| | Insulinase activity (pooled percentage) ^a | |
|---------------------|---|----------|
| | <i>A</i> | <i>B</i> |
| Crude extract | 133 | 53 |
| Acetone precipitate | 179 | 168 |

^a For definition and interpretation, see (1).

actual presence of an inhibitor in this supernatant fluid was readily demonstrated. After the acetone was removed by distillation *in vacuo*, and the residue, consisting largely of lipoid materials, was extracted with water, clear filtrates of the aqueous phase were found to contain the inhibitor, as was demonstrated by their effect in suppressing the activity of potent preparations of insulinase. Within certain limits, the degree of inhibition so produced was observed to be roughly proportional to the quantity of inhibitor added (Table II).

Such an inhibitor has been found in extracts of the livers of all species so far investigated (beef, rat, and rabbit). Muscle extracts of these animals contain considerably less inhibitor, a fact which may be of some

physiologic import in view of the diminished insulinase content of this tissue as contrasted with liver (1). Neither animal nor human blood or urine contained appreciable amounts of this inhibitor.

The inhibitor is quite soluble in water, acetone, 95% ethyl alcohol, and hydrochloric and trichloroacetic acids. It is apparently completely insoluble in ethyl and petroleum ethers, since continuous extraction of active aqueous solutions for as long as 24-48 hr. failed to remove the inhibitor from the aqueous phase.

In acid solutions, the inhibitor was remarkably stable to heat. In solutions adjusted to pH 0.5, the inhibitor activity was not appreciably affected by autoclaving for as long as 30 min. at 15 lb. pressure. Similar

TABLE II

*Insulinase Inhibitor Activity of Acetone-Soluble Fraction
of Beef Liver Homogenates*

All incubations for 30 min. at 37°C. and pH 8.0 in a reaction mixture with total volume of 2 ml. The insulinase was prepared by extracting one part of acetone-dried liver powder with 5 vol. of water at pH 8.0. The centrifuged supernatant was used. The inhibitor was prepared by blending beef liver with 5 vol. ice-cold acetone. The homogenate was filtered through paper and the filtrate concentrated *in vacuo*. The residue was extracted with water and the aqueous phase made up to a volume 1/10 that of the original filtrate and adjusted to pH 8.0.

| Beef liver insulinase ml. | Inhibitor ml. | Insulinase activity (pooled percentage) |
|---------------------------------|------------------|--|
| 1.0 | — | 180 |
| 1.0 | 0.5 | 80 |
| 1.0 | 0.25 | 77 |
| 1.0 | 0.125 | 147 |

treatment at pH 8.0 resulted in considerable loss. The inhibitor was rapidly destroyed merely by standing at room temperature at pH 11.7. Ashing, even in the presence of acid, resulted in a destruction of the inhibitor (Table III).

Solutions of the inhibitor rapidly lost activity when dialyzed in the cold. These properties would tend to characterize the active principles as a non-lipoidal substance of rather low molecular weight whose activity is not dependent on the presence of such linkages as may be destroyed by acid hydrolysis.

Attempts to isolate the active material by its precipitation from aqueous solutions by cations such as Hg^{++} or Cu^+ or by anions such as phosphotungstate have not led to recovery of activity. As a result,

efforts to characterize the nature of the inhibitor or to obtain it in a pure state have not been successful. During some of these attempts, however, an opportunity was afforded to demonstrate that its activity was not altered by treatment with H_2S .

Relatively purified preparations could be obtained by the method used by Grollman for the purification of the antidiuretic substance from urine (4). With this procedure, an appreciable amount of the inhibitor activity of crude trichloroacetic acid extracts of beef liver could be adsorbed on activated charcoal and eluted with glacial acetic acid. A description of a typical preparation will serve to describe the method utilized for this purpose.

Eighteen hundred grams of fresh beef liver was homogenized in a Waring Blendor with 1800 ml. of 10% trichloroacetic acid. The result-

TABLE III

Effect of Heat and pH on Stability of Insulinase Inhibitor

The results represent the effects of variously treated aliquots of the same crude inhibitor preparation on the activity of a beef liver insulinase similar to that described in Table II. After undergoing treatment, the several samples of the inhibitor were readjusted to equivalent volumes and pH 8.0. Insulinase activity was determined in the standard manner.

| Treatment | Insulinase activity (pooled percentage) |
|---|--|
| None | 102 |
| Autoclaving at pH 0.5 for 30 min. | 124 |
| Autoclaving at pH 8.0 for 30 min. | 178 |
| Room temperature at pH 11.7 for 30 min. | 190 |
| Ashing | 199 |

ant mixture was centrifuged and the supernatant filtered through coarse paper. The residue was re-extracted in a similar manner with an equal volume of 5% trichloroacetic acid. The combined supernatants, measuring 3670 ml. were adjusted to pH 4.5. This extract contained no readily demonstrable fat or protein and was free of insulinase but contained considerable inhibitor activity. Thirty-seven grams of norite was added, the whole stirred for 1 hr. and then allowed to stand overnight in the cold. The charcoal was then collected on a Büchner funnel, washed well with distilled water and suspended in 720 ml. of glacial acetic acid. This mixture was stirred for 4 hr. and then centrifuged at a speed high enough to sediment the residue. The supernatant, measuring 650 ml., was collected, and to it was added 6500 ml. of a mixture of equal parts of absolute ethyl alcohol and petroleum ether. On standing,

a small amount of precipitate formed which was collected and dried. By this means, there was obtained 548 mg. of an amorphous powder with a faint tan color which dissolved readily in water to a crystal-clear solution containing marked inhibitor activity.

Attempts to purify the inhibitor by adsorption on other materials such as kaolin, permutit, or cationic or anionic resins were unsuccessful or gave products of much less potency.

On the basis of dry weight, such preparations were appreciably more active than the original trichloroacetic acid extracts. Unfortunately, much of the inhibitor activity of the crude liver was lost during the process of purification. A comparison of the activity of the dry powder, as contrasted to the crude extract in terms of equivalent weights of fresh

TABLE IV

Comparison of Activity of Crude and Purified Insulinase Inhibitors

Beef liver insulinase prepared as described in Table II. The crude inhibitor was prepared by direct extraction of fresh beef liver with trichloroacetic acid as described in the text, concentrated *in vacuo* and adjusted to pH 8.0. The purified inhibitor was prepared from the crude material by adsorption on charcoal, elution with glacial acetic acid, and precipitation by an absolute ethyl alcohol-petroleum ether mixture as described in the text. One ml. of crude inhibitor corresponded to 0.47 g. of fresh liver; 1 ml. of the purified inhibitor corresponded to 1.88 g. of fresh liver. All incubations of reaction mixtures performed for 30 min. at pH 8.0 and 37°C.

| | Insulinase activity (pooled percentage) |
|--|--|
| Beef liver insulinase | 197 |
| Beef liver insulinase + crude inhibitor | 56 |
| Beef liver insulinase + purified inhibitor | 104 |

liver, is found in Table IV. It will be noted that the purified inhibitor was less active than the crude extract although the former had been obtained from four times the quantity of crude liver as was used for preparation of the latter. In the actual assay, 0.28 mg. of the purified inhibitor was added to the reaction mixture as contrasted to the case of the crude extract, with the total quantity of material extractable with trichloroacetic acid from 0.24 g. of liver.

As far as could be determined, the inhibitor does not appear to function as a nonspecific enzyme poison. The purified material exerted no appreciable effect on the activity of crystalline pancreatic trypsin, crystalline pepsin, or the streptokinase-activated proteolytic system (plasmin) of human blood. Examples of this degree of specificity are found

TABLE V

Lack of Effect of Insulinase Inhibitor on Trypsin and Human Plasmin Activity

Trypsin and plasmin activities estimated from amount of acid-soluble "tyrosine-like" material liberated during incubation for 60 min. with a hemoglobin substrate at 37°C. and pH 7.5 as otherwise described by Northrop, Kunitz and Herriott (3). The reaction mixtures consisted of 40 μ g. of crystalline trypsin or an amount of plasmin prepared by the activation of 10 mg. of Fraction III^a of human plasma by an excess of streptokinase. The inhibitor, when present, consisted of 0.5 ml. of the same material used in Table II.

| | "Tyrosine" liberated μ g./ml. |
|---------------------|--------------------------------------|
| Trypsin | 1070 |
| Trypsin + inhibitor | 1113 |
| Plasmin | 285 |
| Plasmin + inhibitor | 276 |

^a Kindly furnished through the generosity of Dr. Dwight Mulford of the Massachusetts Blood Plasma Fractionation Laboratory.

in Table V. Such observations, together with the lack of effect of soy-bean inhibitor and serum on insulinase (1), again emphasize the lack of identity of insulinase and the more common proteinases. It should also be noted that, although the method of its purification is similar to that used for the antidiuretic substance in urine, the two are apparently not identical since urine does not contain appreciable amounts of insulinase inhibitor.

TABLE VI

Effect of Intravenous Injection on Insulinase Inhibitor on Fasting Blood Sugar of the Rabbit

The inhibitor was an aqueous solution of the material purified by elution from charcoal as described in the text. One ml. represented the inhibitor content corresponding to 100 g. of fresh liver.

| Rabbit | Inhibitor injected ml. | Blood sugar | | | | |
|--------|-------------------------------|-------------------------------|----------------------|----------------------|----------------------|-----------------------|
| | | Before injection mg.-% | After injection | | | |
| | | | 30 min. mg.-% | 60 min. mg.-% | 90 min. mg.-% | 120 min. mg.-% |
| 1 | 1.0 | 83 | 83 | 83 | 83 | 83 |
| 2 | 1.0 | 88 | 92 | 92 | 92 | 92 |
| 3 | 10.0 | 87 | 85 | 86 | 98 | 108 |
| 4 | 10.0 | 86 | 85 | 87 | 87 | 97 |

In view of its marked *in vitro* activity, it was considered of interest to determine the effect of the injection of the insulinase inhibitor on the blood sugar regulatory mechanisms of the rabbit. It was believed that, if a physiological effect could be so produced, it might consist of a suppression of or decrease in the rate of inactivation of insulin within the body. This, in turn, would tend to exaggerate the effect of this hormone and might manifest itself either in a decrease in the fasting blood sugar level or an increase in the sensitivity to exogenous insulin. To investigate this question, the preparations purified by charcoal adsorption were tested and found not to exert any marked evidences of acute toxicity. Rabbits easily tolerated intravenous injections of amounts equivalent to as much as 1 kg. of fresh liver. As is demonstrated in Table VI, the injection of even such quantities produced no effects on the fasting blood sugar of the rabbit other than a late hyperglycemia of moderate proportions, a result opposite to that which might have been anticipated. Similarly, numerous trials failed to reveal any effect of the parenteral administration of the inhibitor on sensitivity to injected insulin as tested by a convulsion technique in mice.

DISCUSSION

The existence of a system which inactivates insulin *in vitro* (insulinase) has been established (1). Of more concern, however, has been the investigation of its role in the regulation of carbohydrate metabolism in the intact animal. The observations of Weisberg, Friedman, and Levine (5) in regard to the probably greater rate of inactivation of insulin by the liver of the intact nonfasted dog and the decrease in this rate of inactivation in the fasted animal are in accord with our findings concerning the distribution of insulinase and with the concept that liver insulinase exerts a physiological effect.

We have attempted to investigate the physiologic significance of insulinase by determining the influence of a variety of factors, known to be associated with metabolic changes, on the activity of standardized rat liver extracts. For this purpose, the effects of fasting and the ingestion of certain types of diets on the insulinase activity of such extracts have already been reported (6,7,8). It is evident, for example, that fasting is associated with a significant decrease in the insulinase activity of these extracts (6).

As a result of the present study, which demonstrates the existence in such extracts, not only of insulinase, but of insulinase-inhibitor as well, it becomes essential to consider the existence of two complementary systems, each of which could be concerned with the biological destruction of insulin, the inhibitor being presumed to act by virtue of its ability to depress the rate of insulin destruction by insulinase. It also appears plausible to consider that the rate of destruction of insulin within the living cell may be determined not necessarily by the effect of its *total* concentrations of insulinase and inhibitor but rather by the *balance* of activity made available by the physiological mobilization, to varying degrees, of either of the two systems. The assays of the insulinase activity of extracts had obviously estimated the total potential activity released after cellular disintegration. That such total activity may not be representative of the actual "working" activity of the cell prior to homogenization is indicated also by the observation that liver slices destroy insulin much less actively than do extracts per unit of tissue mass (9).

The failure of the intravenous injection of large amounts of inhibitor to produce a demonstrable effect on the blood sugar regulatory mechanism of the rabbit cannot be construed as indicating that either the inhibitor or insulinase has no physiological role. As is the case with other substances of assumed physiologic importance, *in vivo* injections may be ineffective (10), either due to their inability to penetrate the cellular membrane or because the cell is already saturated with respect to their activity. At any rate, the failure to induce a pharmacologic effect cannot be due to inactivation of the inhibitor by the blood since the blood of various animals has been found to have no effect on the activity of the inhibitor.

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We wish gratefully to acknowledge the technical assistance of Gladys Perisutti.

SUMMARY AND CONCLUSIONS

1. Liver extracts contain a substance which inhibits the *in vitro* inactivation of insulin by liver insulinase. Muscle extracts contain less of this inhibitor.
2. Some of the properties of this substance and a method for its partial purification have been described.

3. Intravenous injections of large amounts of the inhibitor have no apparent effect on the blood sugar regulatory mechanisms of small animals.

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Metabolism of Soybean Leaves. I. The Sequence of Formation of the Soluble Carbohydrates During Photosynthesis ¹

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INTRODUCTION

The primary purpose of this communication is to present the sequence of formation of the soluble carbohydrates in young soybean leaves (var. Hawkeye).

Considerable interest in this question has existed for decades. It has been studied previously in experiments of at least several hours duration, with indecisive results. Experiments using $C^{14}O_2$ on barley seedlings (2) indicated extensive incorporation, indeed almost an equilibration, of the C^{14} with the soluble carbohydrates within 1 hr. It was shown by Calvin and Benson (8) that radio-sucrose was detectable in *Chlorella pyrenoidosa* within 30 sec., although phosphorylated carbohydrates (as fructose 1,6-diphosphate) were present in amounts containing considerably higher radioactivity prior even to this. Hydrolysis of the sucrose indicated the specific activity of the resulting fructose to be greater than that of the glucose. It was therefore believed that sucrose arose from a condensation between a phosphorylated fructose and free, relatively inactive, glucose. The condensation process was depicted as essentially the reversal of the known pathway of sucrose glycolysis. Similar results were obtained also with geranium leaves and barley seedlings (6,7).

Our results show that the first free sugars formed (15 sec.) in soybean leaves are the trioses, dihydroxyacetone and glyceraldehyde, and glucose. Kinetic extrapolations would indicate that the first sugars are

¹ Contribution No. 94 from the Institute of Atomic Research and the Botany Department. A portion of this work was performed in the Ames Laboratory of the Atomic Energy Commission.

probably the trioses. Sucrose appears in quantity and equivalent specific activity later than the glucose, and is, in turn, followed by raffinose. Within 90 sec. the radioactivity distribution of the carbohydrate mixture assumes a character almost equivalent to that of the leaf which has been photosynthesizing for 15 min. During the periods investigated, phosphorylated sugars (to be reported in detail in the near future) never appear in quantity or with a specific activity higher than the predominant sugar of the particular time interval. There is some indication that with lower light intensities, or shorter time intervals at the higher light intensities, they may become prominent.

METHODS

Feeding of $C^{14}O_2$

Soybeans, var. Hawkeye, were grown in Hoagland's nutrient solution until the second trifoliate leaves were about one third of full size. Depending upon the light and temperature this took 3-4 weeks. Taken into the laboratory, the first trifoliate

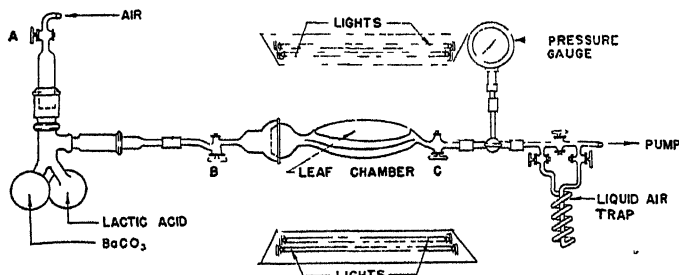


FIG. 1. Apparatus used to generate $C^{14}O_2$ to feed leaves, and to remove excess radioactivity at termination of experiment.

leaves were detached and placed into the leaf chamber, shown in Fig. 1, and permitted to photosynthesize at 3300 ft.-candles for approximately 15 min. In the meantime, 9 mg. of 6% $C^{14}Ba(C^{14}O)_4$ was placed in one arm of the gas-generating chamber, dilute lactic acid in the other, and a slight vacuum drawn. The $C^{14}O_2$ was liberated by rotating the reaction chamber until the lactic acid made contact with the barium carbonate. Following evacuation of the (500 ml.) leaf chamber down to ca. 150 mm., the radioactive carbon dioxide was permitted to enter by the simultaneous opening of stopcocks A and B. Stopcock A was closed just prior to the attainment of atmospheric pressure. At the conclusion of the allotted time the gas in the leaf chamber was drawn through the spiral trap which was immersed in the liquid nitrogen for 5 sec., stopcock C was closed, the main joint disconnected, and the leaf chamber flooded with either liquid nitrogen or an equilibrium mixture of Dry Ice and 80%

ethanol. (The latter has certain conveniences, especially in the dilution of unreacted radioactive carbon dioxide, the use of the same alcohol as solvent for extraction, and the possible unavailability of liquid nitrogen.) The total time from the conclusion of the photosynthesis to destruction of the leaves by freezing was not greater than 10 sec.

It was determined that no highly significant differences in the chemical character of the resulting solution ensued whether extraction was made at 0°C., room temperature, or at the boiling point of the 80% ethanol. Furthermore, in experiments in which the leaves were plunged into liquid air and then into boiling 80% ethanol, the resulting chromatograms were identical with those following the CO₂-ethanol procedure.

Chromatography

Following filtration and washing of the residual leaf tissue, the filtrate was concentrated *in vacuo* to approximately 5 ml. or less, and portions taken for chromatography. A desired portion of the concentrate was passed successively through a cationic exchange column (Nalcite HCR) and an anionic exchange column (IR 4B). The first column adsorbed such cationic materials as amino acids and benzopyrylium compounds (flavones, *etc.*); the second, anionic substances as organic acids and phosphorylated compounds. The nonionic compounds which pass through included the carbohydrates, fats, sterols, *etc.*, but only the first possessed appreciable activity during the course of our experiments. This nonionic fraction was generally concentrated *in vacuo* to a volume of 1 ml. or less and 10–50 μ l. were used per chromatogram.

Our chromatographic chambers were constructed of plywood with central windows inserted for observation. For convenience we used an ascending column, the terminal upper portion of the chromatographic sheet being stapled around a glass rod from which the paper was suspended. For reproducibility we found it advisable to use solvent troughs of identical dimensions ($\frac{1}{2}$ in. \times 2 in. \times 24 in.). Stainless steel was satisfactory but not as good as porcelainized material. The time of ascent varied, according to the solvent, from 24 to 48 hr. In later experiments the chromatography was considerably simplified by reducing the size of the paper to 8 in. square, permitting the use of the 5-lb. wide-mouth bottle ordinarily used for solid chemicals.² The chromatographic paper was formed into a cylinder by stapling, and was inserted within the bottle containing ca. 100 ml. of the solvent. Ascent was complete in 5–6 hr. This diminished time also reduced the diffusion and resulted in more clearly defined spots so that except for the smaller amount of material carried, these chromatograms were in every way at least the equal of the larger sheets.

For carbohydrates we found the following solvent pair to be quite satisfactory: (a) a solution of phenol saturated with water (P), and (b) a solution of butyric acid, butanol, and water (BABW) mixed in the volume ratios of 2:1:1. Ester formation was considerably slower using butyric acid instead of propionic acid. (When using butanol-acetic the character of the solvent is considerably changed in less than 24 hr. by virtue of butyl acetate formation. This is obviously highly undesirable.) It was generally not necessary that the phenol be purified, and technical grades of both butyric acid and butanol appeared to be satisfactory.

² This procedure is used in the laboratory of Prof. D. French of the Chemistry Dept. of this College.

A variety of reagents has been suggested to reveal the locations of the carbohydrates (10). A reagent (14) which we have found very satisfactory was prepared by adding 20 ml. of 10% ammonium molybdate to 3 ml. of concentrated hydrochloric acid with shaking, after which 5 g. of ammonium chloride was added. Any phosphate, or readily hydrolyzable phosphorylated compound on the paper, gave an immediate yellow color due to the formation of the yellow ammonium phosphomolybdates. The paper was heated (75°C.) in a chamber constructed of plywood, lined with sheet aluminum, and coursed with copper tubing through which steam was passed. Within 15 min. the reducing substances on the paper gave a distinct blue spot as a result of the reduction of the molybdate to molybdenum blue. Ketoses were more sensitive to the test than aldoses, although the latter became more prominent on standing at room temperature for a short time after heating. The background was white after the heating, eventually assuming a light blue color, but the location of the spots was never lost. It was possible by this method to determine 5 μ g. of glucose and to detect 2 μ g. Overheating of the chromatogram increased the density of the background. The advantage of this method lay in the observation that the test was given not only by the usual reducing sugars, but also by those nonreducing compounds, as sucrose, which on hydrolysis in the acid medium of the reagent, yielded reducing substances.

For the purposes of degradation it was desirable to have considerable activity, often more than was available in one resolved spot. "Mass-production" was accomplished by the resolution of multiple spots on a single sheet. Although reasonable resolution was obtained by the use of BAW, increased resolution resulted from rerunning the same sheet in the same direction, using phenol (see Fig. 4, center). It has been suggested that, instead of multiple spots, a continuous line of material be used (19).

Following chromatography the spots were located by means of radioautography. For this purpose the trimmed sheets were placed in a 14 in. \times 17 in. cassette in contact with Kodak X-Ray No-Screen film, for a period of hours to days (we generally had about 75,000 disintegrations/min./spot, and permitted exposure for 2 days). For purposes of subsequent alignment, the chromatogram was dotted above and below with a suspension of Ba^{140}O in ink. After development of the x-ray film the spots on the chromatogram were located by superposition on the film and cut out for analytical purposes.

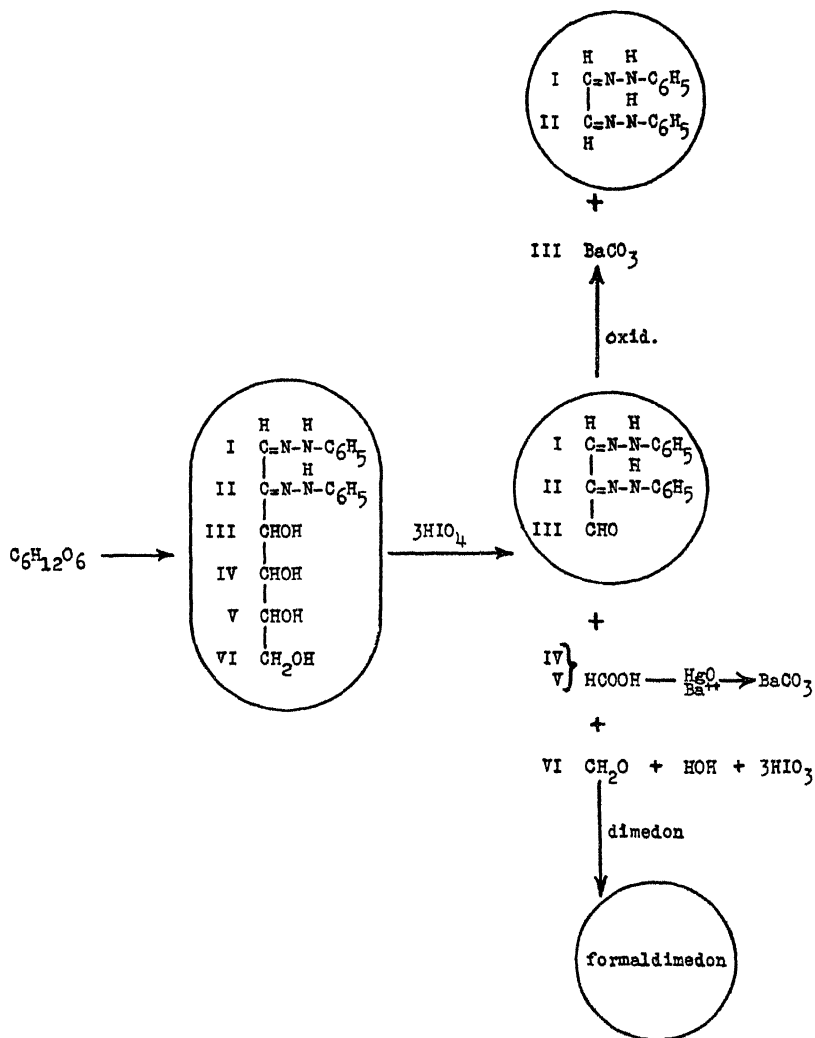
ANALYTICAL METHODS AND RESULTS

The chemical nature of the spot was ascertained initially by association, either homologously with other compounds, by "expected" modifications of excursion values (R_f) during chromatography, or by sheer intuition, guess, or elimination. Following complete coincidence of R_f values on trial runs with inert compounds, the addition of suspected nonradioactive material to the spot prior to chromatography resulted in modification of the size of the subsequent radioactive spot, if the two were identical; if they were not, the radioactive material retained its original dimensions. After agreement of R_f values, the radiomer (the radio compound corresponding to the natural compound) was cut out,

eluted, diluted with the suspected nonradioactive material, converted to a convenient derivative, and recrystallized to constant specific activity. Where the total initial activity was known, the specific activity of the finally recrystallized material bore the proper relationship to the initial amount of inert compound added. For example, assume that to the spot (containing approximately 10 μ g. of presumed radioactive glucose) there were added 100 mg. of inert glucose and 2 ml. water. A 10- μ l. aliquot of this solution indicated the two ml. to contain 10,000 counts/min. The glucose was converted, *e.g.*, to the osazone. Following a series of recrystallizations, after each of which a determination of specific activity was taken until a constant value was obtained, the final weight of osazone was 50 mg. This is equivalent to $(180/358)(50) = 25$ mg. glucose. If the suspected material were glucose the total activity in the osazone should have been $10^{1/4} = 2500$ or 50 counts/min./mg. In this manner, we identified each of the constituents of our carbohydrate fraction.

In addition, as Table I indicates, we degraded certain of the sugars to determine the distribution of activity within the molecule. Analytical methods for degradation of radioactive glucose already exist (3,18), the more quantitative involving bacterial fermentation of the glucose to lactic acid. The lactic acid may be oxidized either to acetic acid and carbon dioxide or to acetaldehyde and carbon dioxide. In both cases the carbon dioxide corresponds to C-3 and C-4 of the glucose. When using the former method the barium acetate is pyrolyzed to acetone and barium carbonate. The residual barium carbonate corresponds to C-2 and C-5 of glucose, while the acetone is subjected to the iodoform reaction (12), the resulting iodoform corresponding to C-1 and C-6.

In our work we initially had no reason to believe that the sugars were composed of two equal halves. For our purposes we therefore adopted the following chemical method which was considerably more rapid, provided information as to the equality of the halves, and, although not as direct as the above degradation, answered the immediate question of the distribution of radioactivity. The method is outlined below: Carbon 6 (= 1) was determined directly as were C-1 + 2 and C-1 + 2 + 3. Carbons 2 and 3 were thus obtainable individually by subtraction, a procedure not as desirable as a direct method, but sufficient for the present purposes. An additional advantage of the method was that all compounds were beautifully crystalline, and of a similar structure which permitted direct comparison of activities of plated material.



Glucose (or fructose or mannose) was converted to glucosazone. By the addition of acetic acid to the normal procedure given for preparation of the osazone the yield was doubled (from approximately 40% to approximately 80%): 500 mg. glucose, 1000 mg. phenylhydrazine hydrochloride, 1500 mg. sodium acetate, 10 ml. water, and 1 ml. acetic

acid were mixed in a test tube which was then placed in a boiling water bath for 15 min. (when using fructose, 10 min. was sufficient). In the case of sucrose, hydrolyzed under optimal conditions (5), the phenylhydrazine hydrochloride and sodium acetate were added directly, and the acetic acid was omitted.

The phenylosazone thus prepared contained almost 10% of adsorbed inert material and where a count was desired at this point, the osazone was recrystallized (50% ethanol-water). The osazone was oxidized with periodate (9), and the osazone of mesoxalaldehyde (C-1 + 2 + 3) filtered out. A similar procedure has been described independently by Topper and Hastings (17). Their procedure was tested with glucose containing activity only in C-3 and C-4 of glucose. Our procedure differs somewhat, and has been verified using synthetic glucose-1-C¹⁴ (16), 90-sec. glucose, and uniformly labeled glucose (from tobacco starch), both by our present procedure and the bacteriological method.

By this method it was shown for the 90-sec. glucose (as for all others) that the activity of C-1 + 2 + 3 equaled one half that of C-1 + 2 + 3 + 4 + 5 + 6. Therefore the activity of C-1 + 2 + 3 equals the activity of C-4 + 5 + 6. Furthermore, by this method we established percentage values of activity for C-1 + 2, C-3, and C-6. By the bacteriological method mentioned above we have shown that in the same glucose sample C-1 + 6 bear the same relation to C-2 + 5 and C-3 + 4 as does C-6 to C-2 and C-3. It was therefore highly probable that C-1 equaled C-6. A direct demonstration of this equivalence is possible by the Wohl degradation given by Topper and Hastings (17) or by degradation of glucose with hydrobromic acid to levulinic and formic acids (15). These procedures we have not yet performed. In the degradation presented here, the C-4 + 5, present as formic acid, have been oxidized by mercuric oxide to carbon dioxide, but the precipitated barium carbonate tended to be about twenty per cent low in activity and of extremely poor yield. Similarly, oxidation of the bisphenylhydrazone mesoxalaldehyde to glyoxalosazone (and carbon dioxide), as given by Diels (11), resulted in a low yield of carbon dioxide containing 6-7% of the initial activity when using synthetic glucose-1-C¹⁴. Considerable effort was expended without avail to improve this oxidation. The use of a variety of oxidizing agents, some of which increased the yield but did not diminish the activity, caused us to rely upon the method outlined above. The bisphenylhydrazone mesoxalaldehyde was isolated from the periodate oxidation of the glucosazone (C-1-6), and represents

C-1 + 2 + 3. It was recrystallized as suggested by Chargaff (9). The filtrate from the oxidation was evaporated *in vacuo* to *ca.* one-tenth its original volume, additional water was added to the residue which was again concentrated *in vacuo*. To the collected distillates was added (for 100 mg. of glucosazone) 200 mg. of dimethyldihydroresorcinol (dimedon) dissolved in 1-2 ml. of ethanol and made up to *ca.* 100 ml. with water. A drop of piperidine was added, and the solution warmed on the steam bath for 10 min. To the solution there was added 3 drops of acetic acid and the mixture was set aside. When the solution had cooled to room temperature, precipitation of formaldimedon was complete. Occasionally recrystallization (from 50% methanol) was necessary to remove a faint yellowish color. This represented C-6 and, as explained earlier, also C-1.

Oxidation in 1% KOH in absolute alcohol of the bisphenylhydrazone mesoxalaldehyde according to Diels (11) resulted in the formation and precipitation of glyoxalosazone. This was readily recrystallized from acetonitrile-water (4:1) in good yield. This derivative is that of C-1 + 2. Having C-1 (the dimedon) with considerable certainty, we have reasonable certainty of the value of C-2 by subtraction from C-1 + 2. Similarly, having C-1 + 2 with equal certainty, we also obtain C-3 by subtraction from C-1 + 2 + 3. All compounds may be readily plated with pyridine.

Molybdenum development of the chromatogram of a commercial sample of dihydroxyacetone (DHA) showed two spots, a diffuse upper spot and a smaller lower spot. These presumably correspond to the monomer and dimer, respectively. The addition of this DHA to the mixture of radioactive sugars, followed by one-dimensional chromatography in BABW, showed complete coincidence of upper and lower spots with previously unidentified spots on the radiogram. In practice, the chromatogram itself had shown reducing activity only for the upper spot. Because of the lesser sensitivity of aldoses to our molybdenum reagent, it was believed possible that some glyceraldehyde could well have been in juxtaposition to the DHA dimer, since chromatography had shown them to have almost identical excursion values. We attempted to determine the amount of glyceraldehyde in the spot by the following procedure. We found that the reaction at room temperature for 2 hr. of 2,4-dinitrophenylhydrazine with glyceraldehyde (\rightarrow phenylhydrazone) and with DHA (\rightarrow osazone) resulted in a fivefold larger yield of phenylhydrazone of the former. A repetition of this reaction with a

mixture of glyceraldehyde (20 mg.), DHA (5 mg.), and radiomer resulted in 36 mg. of phenylhydrazone mixture (predominantly from the glyceraldehyde), the specific activity of which indicated that the initial spot contained no more than 5% glyceraldehyde. Furthermore, the remainder of the phenylhydrazone derivative which precipitated within 24 hr. was, as determined by its melting point, almost pure DHA, and of a specific activity adequate to account for the remaining activity. It must be emphasized that it is probably not valid to infer a

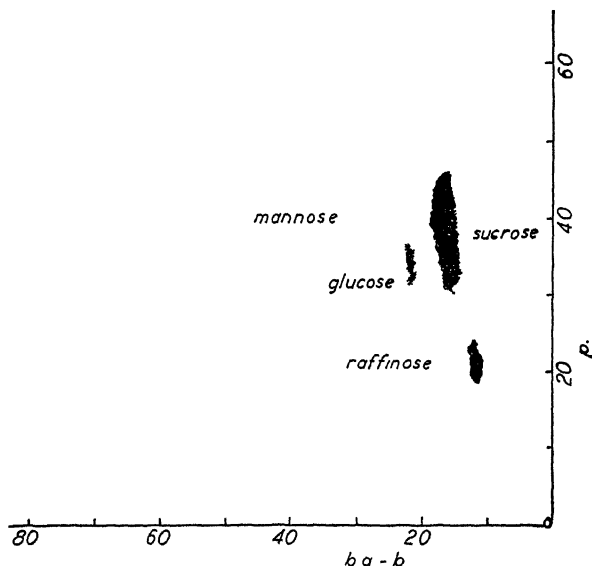


FIG. 2. Radiogram of sugars resulting from 15-min. photosynthesis. The fructose which is just above and between the mannose and glucose is barely discernible. The solvents: butyric acid, butanol, water (b.a.-b.) and phenol, water (p.) were used in the directions indicated.

distribution of trioses on the chromatogram as that occurring in the plant, since the tautomerism is so readily accomplished that the ratio of trioses found may be due solely to the nature of our chromatographic solvents..

Dihydroxyacetone itself, and any accompanying glyceraldehyde, was identified, via the dibenzoyl derivative, readily recrystallized from 95% ethanol, and conformed to the identification standards previously described (constant specific activity on recrystallization, *etc.*).

We ascertained that under our conditions of chromatography, triose phosphate was not hydrolyzed. The triose found is thus believed to be a demonstration of the existence of free trioses in plants during the relatively early stages of photosynthesis. This does not, however, vitiate the possibility that under other conditions, *e.g.*, shorter times or lower light, sugar phosphates may precede free sugars. Experiments designed to elucidate this point are now in progress.

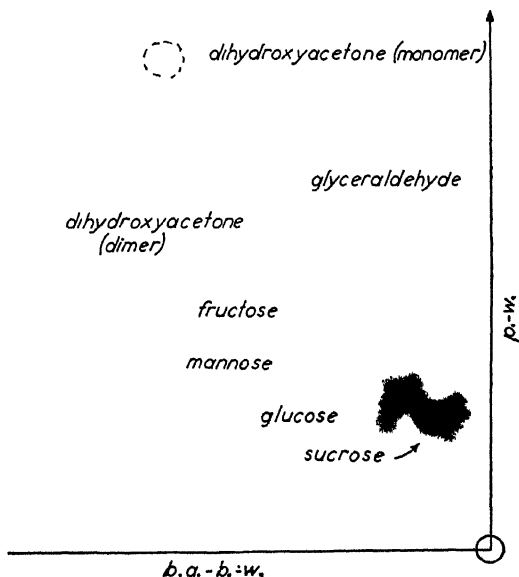


FIG. 3. Radiogram of sugars resulting from 50-sec. photosynthesis. The dihydroxyacetone monomer is lost in photographic reduction.

A typical radioautographed chromatogram (radiogram) for leaves which have synthesized for a long time (2 min. or more; in this instance 15 min.) is shown in Fig. 2. It is seen that the primary sugar, both in quantity (a function of the area of the spot) and radioactivity (density of the spot) is sucrose. Of lesser activity, but greatly exceeding the others, is raffinose, followed by glucose and mannose. (This is not intended to indicate "equilibrium" values. We have, in fact, found conditions under which the most prominent sugars are fructose and glucose. The results are valid for the conditions imposed.) The extent to which the presence of fructose in these experiments results from hydrol-

ysis of sucrose during passage over the highly acidic cation column is not evident here, but it may well account for the major portion of the fructose on the chromatogram. (This could well be avoided in the future by the use of a salt cationic column, rather than one of hydrogen.) Hydrolysis of the sucrose from 90-sec. photosynthesis shows equal activ-

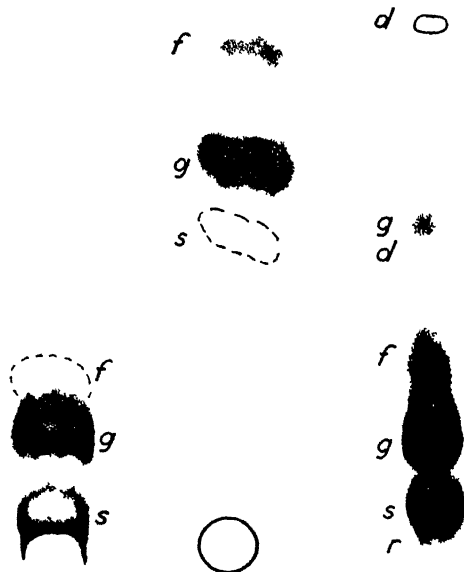


FIG. 4. One-dimensional runs of sugars. Left: Hydrolysis of 50-sec. sucrose. The hydrolysis was intentionally incomplete. *f*, fructose; *g*, glucose; *s*, sucrose. The strip was run in b.a.-b.-w. Center: The same strip rerun in phenol to achieve greater resolution between fructose and glucose. Right: Total sugars from 50-sec. photosynthesis. The dihydroxyacetone monomer (*d*) is present in such small amounts, it is lost in reproduction of the original radiogram where it is quite distinct. Glyceraldehyde (upper *g*) and dihydroxyacetone dimer (lower *d*) are still quite strong (cf. Fig. 5); *f*, fructose; lower *g* (glucose); *s*, sucrose; *r*, raffinose.

ity in the fructose and glucose. Trioses are not present in discernible amounts.

A comparison of the radiogram of 50-sec. photosynthesis (Fig. 3) with that of the longer exposure shows the following major changes: (a) the activity of the glucose is now approximately equal to that of the sucrose, and (b) the trioses form significant fractions of the total amount of activity. The ratio of the specific activity of glucose/triose is 3.0.

The glucose from the hydrolysis of sucrose (Fig. 4) is considerably more active than the fructose.

The striking changes in 15-sec. photosynthesis (Fig. 5) are: (a) the diminution of the activity of sucrose relevant to glucose, and (b) the prominence of trioses, second only to that of glucose. The ratio of the activity of glucose/triose is 2.0. An extrapolation of the relative increase of activity of the trioses from the 50-sec. to the 15-sec. photo-

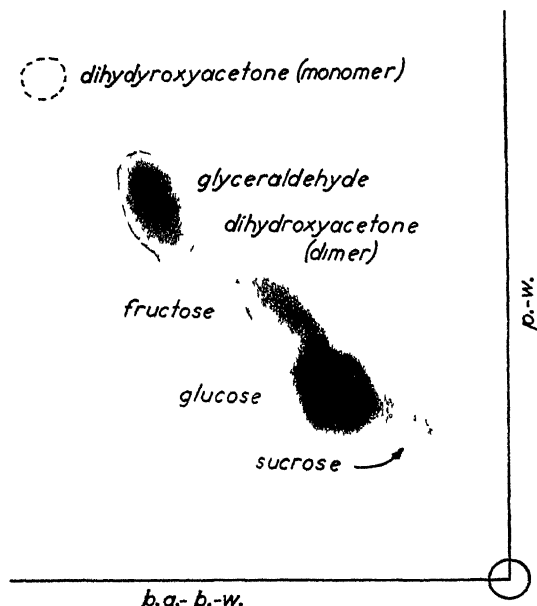


FIG. 5. Radiogram of sugars resulting from 15-sec. photosynthesis. The dihydroxyacetone monomer is lost in photographic reproduction.

synthesis would result in the conclusion that the first sugars formed are the trioses, and these should be the primary sugars at times less than 4 sec.

When the sucrose is hydrolyzed, it is found that the ratio of glucose activity/fructose activity is less in the 15-sec. photosynthesis (2.3) than in the 50-sec. (4.0). In other words, relative to fructose, the *initial* rate of formation of glucose is slower at 15 sec. than at 50 sec.

Degradation studies are summarized in Table I. It is to be noted that the glucose tends to *less* uniform distribution with time, as does the

TABLE I

Distribution of Radioactivity in the Soluble Carbohydrates

C = carbon; e.g., C-3 = carbon no. 3)

| Carbohydrate | Per cent of activity | | | Specific activities |
|---------------------------------------|----------------------|----------------|---------------------------------|-----------------------------------|
| | C-3 | C-2 | C-1 | $\frac{C-1+2+3}{C-1+2+3+4+5+6}$ |
| Glucose-1-C ¹⁴ (synthetic) | 0.0 | 0.0 | 97.8 (C ₆ = 2.2%) | <i>counts/min./mg. C</i> |
| 24-hr. glucose (from tobacco) | 33 | 35 | 33 | |
| 90-sec. glucose | 49 | 32 | 19 | $\frac{177 \pm 7}{170 \pm 7}$ |
| Mannose | 35 | 25 | 30 | $\frac{91 \pm 1}{91 \pm 1}$ |
| 50-sec. glucose | — ^a | — ^a | 21 | $\frac{1450 \pm 24}{1450 \pm 24}$ |
| Sucrose ^b | | | | |
| (a) Glucose | 56 | 26 | 18 | — |
| (b) Fructose | — ^a | — ^a | 24 | — |
| 15-sec. glucose | — ^a | — ^a | 23 | — |
| Sucrose ^c | | | | |
| (a) Glucose | — ^a | — ^a | 27 | $\frac{162 \pm 1}{160 \pm 1}$ |
| (b) Fructose | — ^a | — ^a | 33 | $\frac{71 \pm 1}{71 \pm 1}$ |

^a These values were unfortunately not determined at the time since it was felt that the C-6 (= C-1) values were sufficiently indicative of the distribution so that additional degradation was not warranted. Thus, if C-1 = 33%, then almost certainly C-1 = C-2 = C-3 = 33%. Similarly, if C-1 \neq 33 \pm 2%, the sugar was certainly not uniform, and this was our primary concern. Since then it has become of interest to know whether C-1 = C-2 or not, as on occasion equality may occur. The extent of equality of C-1 and C-2 may be a measure of the amount of "cycling" in the photo-synthetic pathway (see paper No. III for arguments).

^b Ratio of specific activity of glucose/fructose = 4.0.

^c Ratio of specific activity of glucose/fructose = 2.3.

fructose. (This does not imply, of course, that with sufficient time a dynamic equilibrium will not occur. Whether this equilibrium value is that of an equality of radioactivity will depend upon the extent of participation of the "pool" of metabolite in the general metabolism.) Because of the poverty of radioactivity in the free fructose in the various periods, and the known partial hydrolysis of sucrose, it was felt that analysis of the free fructose would not yield significant data. Nevertheless, since the glucose and fructose approach, insofar as the data permit speculation, a uniformity at zero time, it may be significant that the sucrose hydrolysis products from 15-sec. photosynthesis are of more equal specific activities than those of the 50-sec. This is what would be expected from a system where (a) the initial contribution of the free glucose toward sucrose synthesis is negligible and becomes important only in later times, and (b) where the rate of glucose synthesis is considerably more rapid than that of the fructose.

It is apparent that the kinetics of carbohydrate formation are not necessarily simple and that further data are required.

An additional point of interest is the appearance of raffinose without even a trace of galactose, nor is there a discernible amount of other polysaccharide involving, *e.g.*, mannose, which is present in conspicuous amounts in the more lengthy exposures.

Our results are in qualitative and quantitative disagreement with previously published suggestions (8) on the sequence of carbohydrate formation. It may be, however, that different plants have somewhat different paths, not only of carbohydrate formation, but of other substances as well. There is such a desire to look for similarities in organisms that differences tend to be disregarded. There may, indeed, be different sequences in the same plant under different conditions, *e.g.*, age of leaf, time of day, *etc.*

That the glucose formed is nominally not uniform, and that it is composed of two halves of equal radioactivity, indicates that the glucose is almost certainly made from two three-carbon units. Of the two possible condensations: (a) two trioses or (b) glyceric acid and glycerol, the more probable choice is the former. This choice arises both from the absence of significant amounts of glycerol, its esters or ethers until sucrose becomes prominent, as well as the classic demonstration of the coupling of trioses to form hexoses *in vitro*. Such a coupling involves a symmetrical molecule, dihydroxyacetone, which should result in an equality of radioactivity between C-1 and C-3. Earlier investigation

showed that this was not the case (1). The normal explanation thus generally involved the phosphorylated rather than the free triose (8). It has recently been shown, however, that a symmetrical molecule, citric acid, which is generally not considered to be associated in the cell (in the sense of a prosthetic group, or as phosphorylated) does not react symmetrically in subsequent enzymatic reactions. The explanation of this phenomenon involves, among other things, a three-point contact of enzymes with substrate. This is not possible with dihydroxyacetone in precisely the same sense as with citric acid, but it is with glyceraldehyde. This obviously raises the question of the inoperation of isomerase, concerning which one can point to the known frequent inoperation of invertase and various proteolytic enzymes *within* cells. Apart from this, it is possible that those triose molecules involved in a sequence of enzymatic reactions may be held in strong association and passed from one enzyme to another without permitting equilibration. Although phosphorylation is, in fact, a method of passage from one enzyme to another, it is not absolutely required to postulate phosphorylated intermediates, and our glucose may well arise directly from the demonstrated trioses.

A comparison of the activity of the phosphorylated compounds (sugar phosphates and glycerophosphoric acid) with the total activity (4) shows relatively increasing values of phosphorylated compounds with decreasing time, although at this light intensity even in a period as small as 5 sec. they are not the predominant materials. At intervals of 50 sec. or less they are, however, as prominent as the carbohydrates. It is therefore not impossible that they serve as precursors of the hexoses, and more careful study is required to make a choice between alternatives.

ACKNOWLEDGMENTS

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SUMMARY

1. By means of radioactive carbon dioxide it has been shown that under conditions of light saturation the sequence of formation of carbohydrates in young soybean leaves consists of trioses and glucose (trioses probably first), followed by sucrose, then raffinose and mannose.

2. Under all conditions the free hexoses and those obtained from the disaccharides are composed of two equal halves. Because of a nonuniformity of radioactivity within each half of the glucose, that compound must have been derived from two three-carbon units, probably the trioses.

3. The glucose portion of the sucrose is formed prior to the fructose. Fructose from hydrolyzed sucrose shows a more uniform distribution than does glucose from the same moiety. Therefore, the formation of sucrose may follow a pathway different from that of glucose.

4. A rapid method is presented for the determination of the distribution of activity within the hexose molecule and applicable to pentoses and to those polysaccharides which yield hexoses and pentoses on hydrolysis.

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Preparation and Properties of α,α -Di(Acylamino) Aliphatic Acids¹

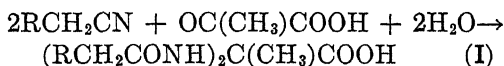
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INTRODUCTION

α,α -Di(acylamino)propionic acids (I) wherein the acyl radical is acetyl (1), chloroacetyl (2), or DL-chloropropionyl (3), have been prepared by the general procedure of treating pyruvic acid in chilled, concentrated sulfuric acid solution with 2 equiv. of the corresponding acyl nitrile, followed by treatment of the reaction mixture with water:



None of these compounds are attacked by preparations of animal or plant tissues. When, however, the α -chloroacyl derivatives are converted to the corresponding α -aminoacyl or α -methylaminoacyl derivatives by treatment with aqueous ammonia or methylamine, respectively (4), the resulting compounds are readily hydrolyzable by tissue preparations to products which include ammonia and pyruvic acid. The only other type of substrates which yield these products by hydrolytic enzymatic reaction are the dehydropeptides [cf. Ref. (5)]

It was considered of interest to extend the preparation of these compounds to include (a) derivatives of α -oxo-aliphatic acids other than pyruvic acid, and (b) asymmetric derivatives of pyruvic acid, and to subject such derivatives to the action of hog kidney extracts.³

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³ Hog kidney extracts were employed because of their considerably greater activity than those of the rat previously used (2-4).

EXPERIMENTAL

The preparation and properties of this novel class of compounds have been fully described in earlier publications (2,3). Except for the case of α -(glycylamino)- α -(acetamino)propionic acid which presents some unusual features, we have therefore not gone into detail in the description of the compounds reported in this paper.

 α,α -Di(chloroacetamino)butyric Acid

Prepared in the same fashion as α,α -di(chloroacetamino)propionic acid (2), except that α -oxobutyric acid (6) was employed instead of pyruvic acid. After crystallization from methyl alcohol the compound melted at 169–70°.

Anal. Calcd.: C 35.4, H 4.5, N 10.3; found: C 35.2, H 4.7, N 10.3.

 α,α -Di(glycylamino)butyric Acid · HCl

Prepared by amination of the above in excess of aqueous ammonia (2). After frequent precipitations from aqueous solutions by absolute alcohol, the compound was freed of adherent ammonium salt.

Anal. Calcd.: C 35.8, H 6.4, N 20.7, Cl 13.2; found: C 35.6, H 6.3, N 20.3, Cl 13.2; m.p. 206° with decomposition.

 *α,α -Di(chloroacetamino)-*n*-valeric Acid*

α -Oxo-*n*-valeric acid (6) was employed.

Anal. Calcd.: C 37.9, H 5.0, N 9.8; found: C 37.6, H 5.2, N 9.8; m.p. 140°.

 *α,α -Di(glycylamino)-*n*-valeric Acid · HCl*

Anal. Calcd.: C 38.2, H 6.7, N 19.8, Cl 12.5; found: C 38.1, H 6.7, N 19.7, Cl 12.6; m.p. 234° with decomposition.

 *α,α -Di(chloroacetamino)-*n*-caproic Acid*

α -Oxo-*n*-caproic acid (7) was employed.

Anal. Calcd.: C 40.2, H 5.4, N 9.4, Cl 23.7; found: C 40.2, H 5.4, N 9.2, Cl 24.0; m.p. 140–1°.

 *α,α -Di(glycylamino)-*n*-caproic Acid · HCl*

Anal. Calcd.: C 40.5, H 7.1, N 18.9, Cl 12.0; found: C 40.5, H 7.1, N 18.7, Cl 12.0; m.p. 206–7° with decomposition.

 α,α -Di(chloroacetamino)isocaproic Acid

α -Oxo-isocaproic acid (6) was employed.

Anal. Calcd.: C 40.2, H 5.4, N 9.4, Cl 23.7; found: C 40.3, H 5.4, N 9.3, Cl 23.2; m.p. 134°.

α,α -Di(glycylamino)isocaproic Acid·HCl

Anal. Calcd.: C 40.5, H 7.1, N 18.9, Cl 12.0; found: C 40.5, H 6.9, N 18.9, Cl 12.0; m.p. 237° with decomposition.

 α -(Chloroacetamino)- α -(acetamino)propionic Acid

To 200 ml. of concentrated sulfuric acid chilled by a Dry Ice-acetone mixture, 35 ml. of freshly distilled pyruvic acid (1 mole) was gradually added with stirring. While maintaining the temperature of the mixture below 5°, 30 ml. of redistilled chloroacetonitrile (1 mole) and 26 ml. of redistilled acetonitrile (1 mole) were successively added. The temperature was then allowed to rise to 30°, and the mixture was stirred at this temperature for 1.5 hr. At the end of this period the reaction mixture was poured in a thin stream over 2 l. of ice shavings with vigorous stirring. The white precipitate which formed was filtered after the ice had melted, washed with cold water, and crystallized twice from methyl alcohol in the form of prisms. The yield was 38.8 g. or 60% of theory.

Anal. Calcd.: C 37.8, H 5.0, N 12.6, Cl 15.9; found: C 37.8, H 4.9, N 12.7, Cl 15.6; m.p. 176°.

If the acetonitrile is added first and then the chloroacetonitrile, no yield is obtained. If the two nitriles are mixed together and added to the solution of pyruvic acid in sulfuric acid, a very small yield of some as yet uncharacterized material is obtained. The melting range of an equal mixture of α,α -di(chloroacetamino)propionic acid (201°) and α,α -di(acetamino)propionic acid (190°) is 185°–90°.

 α -(Glycylamino)- α -(acetamino)propionic Acid

This was obtained by amination in aqueous ammonia in the usual way (2). Melting point 208° with decomposition. After several crystallizations from water-alcohol mixtures, the compound was completely free of chloride. This freedom from halide proves the absence of any α,α -di(glycylamino)propionic acid·HCl and indicates that the original condensation product was as designated and not a mixture of α,α -di(chloroacetamino)propionic acid and α,α -di(acetamino)propionic acid. In any event, amination of such a mixture would have yielded nearly pure α,α -di(glycylamino)propionic acid, for the α,α -di(acetamino)propionic acid, which is alcohol-soluble, would have been removed during the crystallizations.

Anal. Calcd.: C 41.4, H 6.4, N 20.2; found: C 41.1, H 6.4, N 20.1.

 α,α -Di(β -chloropropionylamino)propionic Acid

This was prepared from β -chloropropionitrile and pyruvic acid.

Anal. Calcd.: C 37.9, H 5.0, N 9.8, Cl 24.9; found: C 37.7, H 5.0, N 9.8, Cl 24.5; m.p. 126–7°.

 α,α -Di(β -bromopropionylamino)propionic Acid

This was prepared from β -bromopropionitrile and pyruvic acid.

Anal. Calcd.: C 28.9, H 3.8, N 7.5, Br 42.3; found: C 28.9, H 3.9, N 7.5, Br 42.8; m.p. 136–7°.

α,α -Di(γ -chlorobutyrylamino)propionic Acid

This was prepared from γ -chlorobutyronitrile and pyruvic acid.

Anal. Calcd.: C 42.2, H 5.8, N 9.0, Cl 22.6; found: C 42.1, H 5.9, N 8.9, Cl 22.7; m.p. 112–15°. Attempts to aminate the above three compounds were unsuccessful.

 α,α -Di(acrylamino)propionic Acid

This was prepared from acrylonitrile and pyruvic acid.

Anal. Calcd.: C 50.9, H 5.7, N 13.2; found: C 51.0, H 5.6, N 13.0; m.p. 154–5°.

All of the above compounds are completely stable in aqueous solutions. On the other hand, it has been shown that the related compound, α -(acetamino)- α -(amino)propionic acid, $(\text{CH}_3\text{CONH})(\text{NH}_2)\text{C}(\text{CH}_3)\text{COOH}$, is spontaneously hydrolyzed in aqueous solution to yield equimolar quantities of pyruvic acid and ammonia (2). This compound was prepared by the alcoholysis of the azlactone of α,α -di(acetamino)propionic acid (8). By an analogous procedure, α -(chloroacetamino)- α -(amino)propionic acid has been prepared from the azlactone of α,α -di(chloroacetamino)propionic acid.

Azlactone of α,α -Di(chloroacetamino)propionic Acid

A mixture of 22.3 g. of α,α -di(chloroacetamino)propionic acid (2) and 100 ml. of acetic anhydride was heated with exclusion of moisture on a steam bath for 2 hr., or until the compound completely dissolved. The excess acetic anhydride was then removed *in vacuo* under dry nitrogen, the sirupy residue dissolved in 30 ml. dry ethyl acetate and filtered. On chilling, the azlactone crystallized. It was crystallized again from ethyl acetate in the form of white prisms. Yield 13 g.

Anal. Calcd.: N 11.7, found 11.8; m.p. 112°.

 α -(Chloroacetamino)- α -(amino)propionic Acid

Nine grams of the above azlactone was dissolved in 90 ml. of carefully dried methyl alcohol. The solution was mechanically shaken for 6 hr. at 23°, during which period the desired compound separated in long, narrow plates. The yield was 5.5 g.

Anal. Calcd.: C 33.2, H 5.0, N 15.5, Cl 19.6; found: C 33.2, H 5.1, N 15.5, Cl 19.6; m.p. 113° with decomposition.

Attempts to replace the chlorine by an amino group, by treatment of the compound with either aqueous or liquid ammonia, were unsuccessful.

This compound, like the α -(acetamino)- α -(amino)propionic acid previously studied (2), is unstable in aqueous solution, and spontaneously decomposes to products which include ammonia and pyruvic acid in equimolar proportions. This instability is characteristic of this type of compound wherein a free amino group is attached to the tertiary carbon atom. When this amino group is acylated, the compounds are entirely stable in aqueous solution.

Enzymatic Studies

None of the compounds derived from any α -oxo-acid other than pyruvic acid was found to be enzymatically susceptible under any con-

ditions, and even for compounds derived from pyruvic acid, at least one of the acyl radicals must contain a free amino group [cf. (3)]. These findings suggest a relatively narrow range of specificity for the enzyme or enzymes active on this class of compounds. This range is apparently much narrower than the dehydropeptidases which include systems which are (a) quite active against substrates derived from α -oxo-acids other than pyruvic (9), and (b) quite active against substrates completely lacking an α -amino group, *e.g.*, acetyldehydroalanine (5). No further attention will therefore be paid to the resistant substrates, and subsequent discussion will be concerned with susceptible types of substrates.

α,α -Di(glycylamino)propionic Acid·HCl

Previous studies (2) of the enzymatic hydrolysis of this compound reveal that kidney extract possessed the highest activity among the tissues investigated. When rat kidney extracts were employed as a source of enzyme, equivalent amounts of ammonia and pyruvic acid appeared in the digest, and a consideration of the possible modes of splitting of the compound was made with this observation in view. From the evidence available at that time it was not possible to determine whether initial enzymatic attack took place at a peptide bond, or at a tertiary carbon-nitrogen linkage. In an attempt to shed further light on this problem additional studies have been carried out employing hog kidney extracts as a source of enzyme. The freshly obtained tissue was allowed to thaw, ground in a mortar with sand, extracted with 3 vol. of cold water and lightly centrifuged. The supernatant was dialyzed for 4-6 hr. against cold distilled water. Digests consisted of 1 ml. of extract, 2 ml. of 0.15 *M* borate buffer at pH 8.0, and 1 ml. of substrate solution containing 10-25 μ moles of α,α -di(glycylamino)propionic acid·HCl. Hydrolysis was determined by simultaneous estimations of ammonia and pyruvic acid in duplicate tubes according to methods which have been previously described (2). Recovery experiments were performed by adding known amounts of sodium pyruvate to the tissue extracts. After prolonged incubation, 85-95% of the added pyruvate could be recovered in every case. The chemical determinations of pyruvate were frequently checked by enzymatic determinations with lactic dehydrogenase [cf. (10)] and complete concordance was noted.

Hog kidney extracts exhibit an activity of 10 μ moles/hr./mg. N toward α,α -di(glycylamino)propionic acid. The bulk of the enzymatic activity was concentrated in the sediment after centrifugation for 2 hr. at 26,000 $\times g$. In contrast to the observations made when rat kidney extract was employed, ammonia appeared in the digests faster than did pyruvic acid, and a maximum of 2 moles of ammonia and 1 mole of pyruvic acid per mole of substrate was produced. This phenomenon may be the result of the relatively rapid desamidation of glycine amide by extracts of hog kidney. These observations, however, offer no evidence as to the site of the initial splitting of the compound. Either the concept of primary splitting at the tertiary carbon-nitrogen bond, yielding glycyldehydroalanine and glycine amide, or the initial hydrolysis of a peptide bond, yielding glycine and α -(glycylamino)- α -(amino)propionic acid, is consistent with the observations.

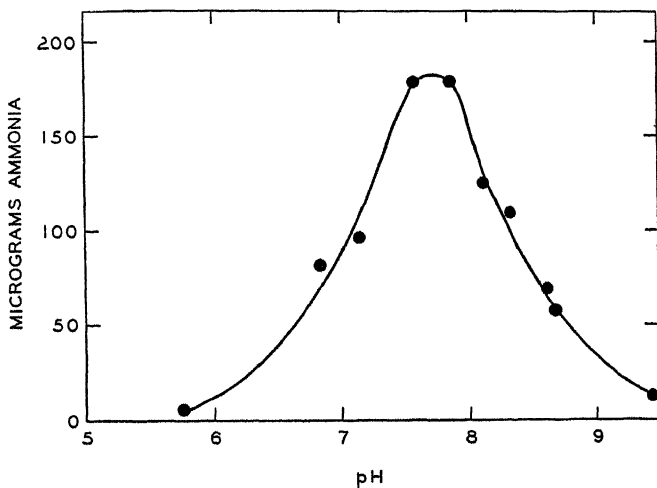


FIG. 1. Relation of pH to the hydrolysis of α -(glycylamino)- α -(acetamino)propionic acid. Digests consisted of 25 μ moles of substrate in 1 ml. of solution; 1 ml. of aqueous extract of hog kidney contained about 1 mg. protein N and 1 ml. of veronal buffer. Temperature 37°. One-hour incubation.

α -(Glycylamino)- α -(acetamino)propionic Acid (II)

This compound proved to be hydrolyzed by hog kidney extracts to products including pyruvic acid and ammonia at a rate of 4 μ moles/

hr./mg. N.⁴ The hydrolysis is maximal at pH 7.6–7.7 (Fig. 1).⁵ The peptide, which possesses an asymmetric carbon atom, exhibits no optical activity and is a racemic mixture. This view was borne out by the observation that enzymatic hydrolysis proceeds smoothly until one-half mole of pyruvic acid is produced per mole of substrate (Fig. 2), suggesting that only one of the two optical enantiomorphs was readily susceptible to hydrolysis by hog kidney extracts.⁶

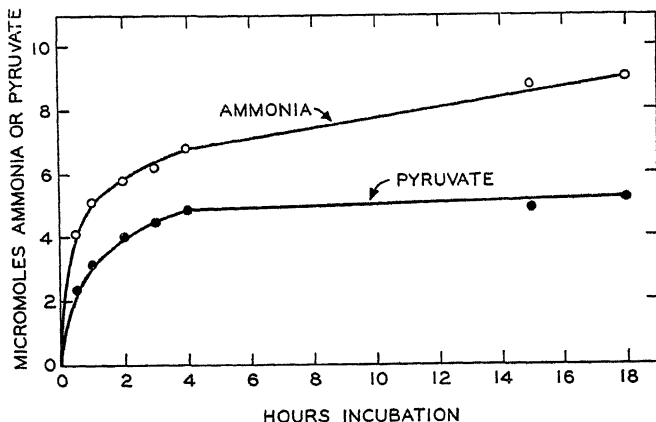


Fig. 2. Relation of length of digestion to the extent of hydrolysis of α -(glycylamino)- α -(acetamino)propionic acid. Digests consisted of 10 μ moles of substrate in 1 ml. of solution; 1 ml. of dialyzed hog kidney extract containing about 2 mg. protein N and 1 ml. of borate buffer at pH 8.0. Temperature 37°.

As in the case of its di(glycyl) analog (2), the bulk of the enzymatic activity in hog kidney toward this substrate was concentrated in the sediment after centrifugation at $26,000 \times g$ for 2 hr.

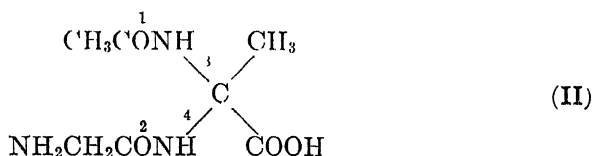
In the course of digestion with hog kidney extracts, ammonia was produced at a greater rate than pyruvic acid, the ratio approaching 2:1 after prolonged incubation (Fig. 2). Since glycine was entirely stable under the experimental conditions, the α -amino group of the glycine

⁴ Rat kidney aqueous extracts hydrolyze α -(glycylamino)- α -(acetamino)propionic acid at pH 8.0 and 37° at a rate of about 2.5 μ moles/hr./mg. N; comparable rat liver and spleen extracts exhibit activities of less than 1.

⁵ The maximum rate of hydrolysis of α , α -di(glycylamino)propionic acid with rat kidney extracts occurs at pH 8.0 (2).

⁶ With a large amount of enzyme, a slow but definite hydrolysis of the second enantiomorph could be observed.

residue presumably did not contribute to the ammonia evolved during the enzymatic digestion. In this case, the observed molar ratios of ammonia to pyruvic acid indicate that ammonia is furnished by both nitrogen atoms attached to the tertiary carbon atom:



The suggestion that the di(acylamino)propionic acids may be physiological precursors of dehydropeptides has been advanced (2). This suggestion envisages primary enzymatic splitting of a tertiary carbon bond yielding a dehydropeptide which is subsequently hydrolyzed to products including equivalent quantities of ammonia and pyruvic acid. In the case of α -(glycylamino)- α -(acetamino)propionic acid (II), initial splitting of bond 3 would yield glycyldehydroalanine and acetamide. Since the latter was found to yield no ammonia under the experimental conditions, and the former yields equal quantities of ammonia and pyruvic acid (5), this hypothesis is not in accord with the observed 2:1 ratio of ammonia to pyruvic acid (Fig. 2).

If, however, bond 4 is the primary point of attack, glycine amide and acetyldehydroalanine are produced. In digests with hog kidney extracts, the former yields 1 mole of ammonia, the latter 1 mole of ammonia and one of pyruvic acid. This mode of splitting thus would yield the observed ammonia to pyruvate ratio. The appearance of pyruvic acid in this case is dependent on the enzymatic hydrolysis of acetyldehydroalanine. Crude hog kidney extracts hydrolyze both α -(glycylamino)- α -(acetamino)propionic acid and acetyldehydroalanine at a rate of about 4 μ moles/hr./mg. N. A 15-fold increase of activity toward α -(glycylamino)- α -(acetamino)propionic acid together with a considerable decrease of activity toward acetyldehydroalanine was achieved as follows: 300 g. of fresh frozen hog kidney was thawed and ground in a Waring Blendor with 3 vol. of cold water. The mixture was strained through gauze, and centrifuged at $600 \times g$ for 20 min. at 0° , and the pellet discarded. The supernatant was thereupon centrifuged at $9,000 \times g$ for 30 min., and the sediment once more discarded. The supernatant was then centrifuged at $26,000 \times g$ at 0° for 2 hr., the supernatant discarded, and the sediment suspended in water. The

resulting suspension contained 2 mg. protein N/ml. The pH of the suspension was brought to 8 by the addition of solid NaHCO_3 , and 1 mg. of crystalline trypsin was added per 100 mg. of protein. The mixture was incubated at 25° for 12 hr., and then dialyzed against distilled water for 12 hr. at 0° . Centrifugation at $26,000 \times g$ for 2 hr. was again carried out, and the sediment was resuspended in distilled water. The resulting preparation hydrolyzed α -(glycylamino)- α -(acetamino)-propionic acid at a rate of $60 \mu\text{moles/hr./mg. N.}^7$ Acetyldehydroalanine was hydrolyzed by the same preparation at a rate of less than $1 \mu\text{mole/hr./mg. N.}$ The latter peptide would thus appear to be ruled out as an intermediate in the enzymatic hydrolysis of α -(glycylamino)- α -(acetamino)propionic acid by hog kidney extracts.

TABLE I

Maximum Ammonia and Pyruvic Acid Yielded by Incubation of 10 μMoles Substrate with Dialyzed Hog Kidney Extracts^a

| Substrate | Ammonia μmoles | Pyruvate μmoles |
|--|------------------------------|-------------------------------|
| α, α -Di(glycylamino)propionic acid $\cdot \text{HCl}$ | 20.0 | 9.5 |
| α -(Glycylamino)- α -(acetamino)propionic acid | 9.0 | 4.7 |
| α -(Amino)- α -(acetamino)propionic acid | 13.2 | 9.5 |

^a Twelve-hour period of incubation at 37° . Incubation of these substrates with rat kidney, liver, or spleen extracts yielded 1:1 ratios of ammonia to pyruvic acid.

On the basis of these observations, it is probable that the substrate is initially split at one of the two peptide bonds. If bond 1 is hydrolyzed, acetic acid and α -(glycylamino)- α -(amino)propionic acid would be produced. The latter is presumably unstable, breaking down to glycine amide, ammonia, and pyruvic acid. If bond 2 is attacked, the first products would be glycine and α -(amino)- α -(acetamino)propionic acid. The latter compound decomposes spontaneously in aqueous solution to yield 1 mole of ammonia and one of pyruvic acid (2). But in the presence of hog kidney extracts, yields considerably higher than 1 mole of ammonia are obtained. Inasmuch as acetamide is not enzymatically split, this indicates enzymatic hydrolysis at the peptide bond as well as spontaneous splitting of the tertiary carbon linkage (Table I).

⁷ The purified enzyme preparation was nearly free of activity toward glycine amide, and therefore in the initial stage of the hydrolysis of this substrate the ratio of ammonia to pyruvate was very nearly 1:1. Only much later in the course of the reaction did the second mole of ammonia slowly make its appearance.

Employing the purified enzyme preparation described above, which exhibits only minimal activity toward glycine amide, digests were prepared consisting of 20 μ moles of substrate, 1 ml. of enzyme suspension and 1 ml. of borate buffer at pH 8. After incubation at 37° for 1 hr., the components of the digests were determined by means of paper chromatography by Dr. Herbert Sober. Both glycine and glycine amide were readily identified in the digest, lending support to the thesis that both peptide bonds are hydrolyzed. If primary splitting at bonds 3 or 4 is ruled out, glycine amide could only arise by initial hydrolysis of bond 1, with subsequent splitting of an unstable intermediate to glycine

TABLE II

Increments of Ammonia and Pyruvic Acid After Treating Enzymatic Digests of α -(Glycylamino)- α -(Acetamino)Propionic Acid with Citrate Buffer at pH 3 and at 100° for 30 Min^a

| Period of enzymatic digestion | Ammonia | | Pyruvic acid | |
|-------------------------------|-------------------|-----------------|-------------------|-----------------|
| | Unheated controls | Heated mixtures | Unheated controls | Heated mixtures |
| <i>min.</i> | μ moles | μ moles | μ moles | μ moles |
| 30 | 4.8 | 7.3 | 3.5 | 6.3 |
| 90 | 7.5 | 10.0 | 5.8 | 7.5 |

^a Digests consisted of 25 μ moles substrate in 1 ml. solution, 2 ml. borate buffer at pH 8.0, and 1 ml. of hog kidney extract. After period of incubation, 3 ml. of citrate buffer was added and the mixture was heated for 30 min. at 100°. Final pH 3.1-3.3.

amide, ammonia, and pyruvic acid. On the other hand, glycine would be a product of initial hydrolysis of bond 2.

Inasmuch as it would appear that primary splitting occurs at one of the peptide bonds, the appearance of ammonia and pyruvic acid would be dependent on the spontaneous or enzymatic hydrolysis of such intermediates as α -(glycylamino)- α -(amino)propionic acid and α -(amino)- α -(acetamino)propionic acid. If these intermediates hydrolyze less rapidly than they are formed, they would accumulate during enzymatic digestion. The observed appearance of ammonia and pyruvic acid would thus be limited by the hydrolysis of the intermediates.

That this is actually the case in the enzymatic hydrolysis of α -(glycylamino)- α -(acetamino)propionic acid is suggested by the following

experiment. Enzymatic digests at pH 8 were brought to pH 3 by the addition of citrate buffer, and heated at 100° for 30 min. This treatment quantitatively hydrolyzed α -(amino)- α -(acetamino)propionic acid, while α -(glycylamino)- α -(acetamino)propionic acid, as well as glycine amide, glycine, and acetamide are not hydrolyzed. After treatment at pH 3 for 30 min. at 100°, the enzymatic digests of α -(glycylamino)- α -(acetamino)propionic acid showed increments of ammonia and pyruvic acid over the unheated controls (Table II), indicating that some intermediate or intermediates accumulated during enzymatic digestion which were hydrolyzable under these conditions. The identification of the intermediates is at present under investigation.

ACKNOWLEDGMENT

The authors are indebted to Mr. Robert Koegel for the elemental analyses.

SUMMARY

1. Several α,α -di(acylamino)aliphatic acids were synthesized by reaction between various acyl nitriles and α -oxo-acids. Only those compounds derived from pyruvic acid, and which contained at least one free amino group on the acylamino radicals were hydrolyzed by tissue enzyme preparations. The products of hydrolysis of the susceptible compounds include ammonia and pyruvic acid.

2. Incubation of α,α -di(glycylamino)propionic acid with hog kidney preparations led to the liberation of close to 2 moles of ammonia and one of pyruvate per mole of substrate.

3. The unsymmetrical α -(glycylamino)- α -(acetamino)propionic acid was hydrolyzed at a maximum rate at pH 7.6–7.7 and to a maximum value of 1 mole of ammonia and 0.5 mole of pyruvate per mole of substrate, indicating ready hydrolysis of only one of the two optical enantiomorphs. Studies on the possible modes of initial enzymatic attack by hog kidney extracts on this compound suggest that the latter is first hydrolyzed at one of the peptide bonds, and that the formation of a dehydropeptide intermediate is not apparently likely.

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Comparison of the Iron-Binding Activities of Conalbumin and of Hydroxylamidoproteins

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INTRODUCTION

Conalbumin and siderophilin, two proteins occurring in egg white and mammalian plasma, respectively, have in common the ability to give stable colored complexes with iron above pH 6. The mechanism of this remarkably specific and firm linkage had been under investigation in this laboratory when an interesting hypothesis was proposed for it by Fiala and Burk (1). These authors compared the siderophilin-iron complex with the well-known colored complexes given by hydroxamic acids ($R\text{-NOH-CO-R'}$) with iron in acid solution. They found that hydroxylamine, in the presence of bicarbonate, bound iron at neutrality, presumably by forming *N*-hydroxycarbamic acid ($R=H$, $R'=OH$). Since traces of carbonate had been found necessary for maximal color development in the reaction of conalbumin or siderophilin with iron (2), Fiala and Burk suggested from analogy that these proteins might contain a hydroxylamino group that would combine with carbonate to produce the iron-binding hydroxycarbamic group ($R=\text{protein}$, $R'=\text{OH}$).

The work of this laboratory had revealed a number of facts which were in discord with this hypothesis. It nevertheless appeared indicated to prepare model proteins containing the postulated hydroxylamino groups. This was possible by treatment of proteins, or preferably esterified proteins, with hydroxylamine, which led to the formation of hydroxylamidoproteins ($R=H$, $R'=\text{protein}$). Such protein derivatives were found to give colored complexes with iron at neutrality. This finding seemed to represent indirect evidence favoring the hypothesis that

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conalbumin might also be a hydroxylamidoprotein. This is a hypothesis similar to that of Fiala and Burk and it is not contradicted by as many of our earlier findings as the hypothesis proposing the addition of carbon dioxide to a hydroxylamino group (1). However, a more detailed study has shown that the hydroxylamidoproteins differ in their mode of iron-binding from conalbumin in almost every respect. A thorough analytical search also has failed to reveal the presence of hydroxylamino or alkylhydroxylamino groups of any kind in conalbumin and siderophilin, even though sufficiently sensitive methods were available. It is with some regret that we feel forced to reject the persuasively simple hypothesis of Fiala and Burk, and to suggest in its stead the much less clearly defined concept that the iron is bound by a suitable steric arrangement of certain amino, carboxyl, phenolic, and possibly other typical protein groups (3). The specificity and stability of linkage appear due not to any unusual component in the system, but only to the appropriate peptide-chain-folding of the native protein, to achieve spatial juxtaposition of the active set of typical protein groups.

EXPERIMENTAL

The method of isolation and assay of conalbumin will be described in detail elsewhere (3). The principle of the assay method was to determine first the maximal color that an unknown sample gave upon addition of iron at pH 7.6, and then to determine the amount of excess iron (noncomplex-bound) in the same reaction mixture by means of phenanthroline (4). A combination of filters (Corning No. 038 and Pyrex No. 554) was used for all conalbumin and phenanthroline readings. From the first reading the conalbumin activity could be ascertained by comparison with a standard curve prepared with pure conalbumin; the second reading yielded by difference absolute values concerning the amount of iron stably bound.

Ovomucoid and lysozyme were prepared according to the methods developed in this laboratory (5,6). Bovine serum albumin (BSA) was a commercial preparation.

Role of Carbonate in Chromogenic Activity of Conalbumin

Schade and co-workers (2) found carbonate in equimolar amounts essential for full color development of the siderophilin-iron complex. In partial confirmation, we have found the presence of added bicarbonate

to favor color development in various buffer media, most notably in phosphate. However, repeated experiments aiming at complete exclusion of carbonate from the system have consistently yielded colored complexes, the intensity of color varying from 30–80% of the maximal in different buffer media.

Probably the most conclusive experiments were performed as follows: to 15–20 mg. conalbumin, dissolved in 3.8 ml. boiled water, was added (in a Thunberg tube with an additional side arm equipped with stopcocks and suitable for direct reading in a Klett colorimeter) 0.2 ml. 3 *M* acetic acid and 2 ml. standard iron solution (28 μ g. Fe in 0.001 *M* sulfuric acid). The main side arm contained sodium hydroxide pellets and a few drops of water. The tube was cooled, evacuated, and held *in vacuo* for 18 hr. Since the protein solution has a pH of 3.2 any CO₂ may be assumed to be released and bound by the alkali. The tube was then either filled with CO₂-free air, or held under vacuum during the development of the color. A mixture of concentrated ammonia and 5 *N* sodium hydroxide was added to the other side arm (separated by a closed stopcock from the main vessel), which was then briefly evacuated causing a vigorous flushing of the small air space with ammonia. After 30 min. the stopcock was opened and ammonia gas permitted to diffuse into the colorless acidic conalbumin-iron solution, which was agitated at intervals. After several hours, as the pH in the latter solution rose to above 7, there appeared the typical conalbumin color; a maximum Klett reading of 60–80 was usually reached within 1–2 hr. Addition of about 15 mg. of sodium carbonate at the time of maximal color development caused an increase of about 10%.

Method of Preparation of Hydroxylamidoproteins

The proteins were esterified with 0.1 *N* methanolic HCl (7). With ovomucoid and lysozyme which are very resistant to denaturation the reaction was performed for 24 hr. at 23°; with bovine serum albumin (BSA) for 5 days at –10°. The protein ester hydrochlorides, isolated either by dialysis or by precipitation and washing with cold ether, were treated with an aqueous solution of hydroxylamine HCl (20%), adjusted to faint phenolphthalein-pink (pH 9) with 5 *N* NaOH, and held for 24 hr. at 23°. They were then thoroughly dialyzed. Ovomucoid and lysozyme gave largely water-soluble products,² BSA was in good part rendered water-insoluble, but was soluble with a trace of alkali.

Direct treatment of the proteins with hydroxylamine at pH 9, without preliminary esterification, led to the introduction of smaller amounts of hydroxylamine, even if the reaction was performed at elevated temperature (53°, 18 hr.). Hydroxylamidolysozyme has previously been prepared by a similar technique by Dr. S. R. Dickman of the University of Utah (personal communication). Table I contains analytical data concerning various preparations.

² Esterification inactivated both proteins (8,9) and introduction of hydroxylamido groups caused further loss of activity in the case of lysozyme (7% was reduced to 0.5% of original activity) but not in case of the trypsin-inhibiting activity of ovomucoid (20% active).

TABLE I
Preparation of Hydroxylamidoproteins

| Protein | Conditions of | | Hydroxylamidoproteins ^a | | | |
|------------------|----------------|------------------------|------------------------------------|--------------------|-----------------------------|--|
| | Esterification | Hydroxyl- amidation | | Water- soluble | Bound NH ₂ OH | Reactive CO—NH ₂ OH (as NH ₂ OH) |
| BSA ^c | —10°, 4 days | °C. | hr. | % | % | % |
| | 0°, 20 hr. | 23 | 18 | 40 | 0.75 | 0.46 (0.54) |
| | 23°, 14 hr. | 23 | 18 | 26 | (0.60) | |
| | None | 23 | 18 | 1 | (1.67) | |
| Ovomucoid | None | 53 | 14 | 7 | (0.23) | |
| | 23°, 18 hr. | 23 | 18 | 78 | 1.33 | 1.20 |
| | 23°, 2 days | 23 | 18 | 70 | 1.39 | 1.07 |
| | None | 53 | 18 | (100) ^b | | 0.28 |
| Egg albumin | None | 23 | 18 | 69 | 0.14 | |
| | None | 35 | 18 | 60 | 0.22 | |
| | None | 53 | 18 | 33 | 0.57 | |
| Lysozyme | 23°, 2 days | 23 | 18 | 66 | 0.63 | 0.38 |

^a Figures represent weight percentages of laboratory-conditioned protein; those in parentheses are for the distilled-water-insoluble fractions, dissolved with a small amount of alkali.

^b Half of the protein became dialyzable in the course of the reaction, and was thus lost. After 3 days of reaction at pH 9, 90% of the protein was lost during dialysis.

^c Bovine serum albumin.

Method of Assay of Hydroxylamidoproteins

Two methods for the determination of hydroxylamido groups in proteins were available. Hydrolysis with 3 *N* sulfuric acid at 105° liberated hydroxylamine which could be oxidized to nitrite and determined by the extremely sensitive method of Csaky (10). One-half to 5 μ g. NH₂OH (about 0.1 μ equiv.) could be determined by this method. Recovery experiments and time series of hydrolysis indicated continuous destruction of hydroxylamine of similar rate in the presence of all proteins studied. Hydroxylamine was apparently rapidly liberated from soluble hydroxylamido proteins, since the highest values were obtained after the shortest hydrolysis time used (1 hr.), but insoluble proteins were hydrolyzed 3–6 hr. All values were corrected from a standard time-destruction curve.

The other method was based on the ability of hydroxamic acids in general to give colored complexes with ferric chloride in hydrochloric acid. Thus from 1 to 5 mg. protein containing 0.2–2 μ equiv. of —CO—NHOH, was treated with 3.1 ml. water, 0.2 ml. of a 3 *M* pH 6 acetate buffer, 1 ml. 2.5 *N* HCl, and 1 ml. 5% ferric chloride in 0.1 *N* HCl. Succinylhydroxylamine, prepared according to Lippmann and Tuttle (11),

was used as a standard. The color was read after 5–10 min. with a dark-green filter against the yellow blank containing all but the hydroxamic acid compound. Good agreement was obtained if the proteins were assayed at several levels, but their chromogenic activity was regularly about 20% lower than the hydroxylamine analysis (corrected for destruction) indicated. In the case of hydroxylamido lysozyme, only 50% of the expected color was obtained. These differences may be due to the nature of the acyl substituent (succinyl in the case of the standard, protein-glutamyl predominating in the average protein, but protein-aspartyl in lysozyme).

Principally this same method was used in the search for alkylhydroxylamine groups ($R-CH_2-NHOH$) in protein, since such groups, even after hydrolytic liberation, might not be expected to be detectable by the method of Csaky. It was found that at low concentrations of hydroxylamine succinylation was not quantitative under the conditions used above. However, a constant proportion of the hydroxylamine (84%) was acylated when 10–50 μ g. hydroxylamine in 2 ml. water, buffered with 0.2 ml. pH 6 acetate, was incubated for 1 hr. with a freshly saturated aqueous solution of succinic anhydride. Addition of 1 ml. water, and hydrochloric acid and ferric chloride as above, permitted the colorimetric determination of hydroxylamine. As expected, *N*-ethylhydroxylamine (prepared by reduction of nitroethane) yielded a chromogenic hydroxamic acid after such succinylation. The hydroxylamidoproteins gave, as expected, no more color when so treated with succinic anhydride, than when directly treated with acid and iron.³ Conalbumin, when analyzed without hydrolysis was precipitated during the test, but the characteristic color was absent from both the precipitate and the supernatant. No precipitation occurred if the protein was digested with pepsin, or with pancreatin after heat denaturation, or partly hydrolyzed with acid. Yet, no color appeared when as much as 50 mg. was analyzed, with or without the addition of succinic anhydride.⁴ This indicates the absence of $-NHOH$ or $-CO-NHOH$ groups from this protein.

Reaction of Hydroxylamidoproteins and Simple Hydroxamic Acids with Iron at Neutrality

When hydroxylamidoproteins (3–30 mg. in 7 ml.) are treated with iron (14–56 μ g.) under the same conditions which favor the conalbumin-iron interaction (pH 7.6, 0.05 *M* phosphate, 0.01 *M* carbonate) a yellow color appears immediately which is of similar intensity (when read with the same filters), as that given by the same weight of conalbumin.

Model experiments with succinylhydroxylamine show that this is a general reaction and that hydroxamic acids bind iron in dilute solution

³ This shows that the excess of hydroxylamine has been quantitatively removed by dialysis. Apparently an appreciable fraction of the bound hydroxylamine is liberated in the course of the Csaky procedure (by the acetic acid used as solvent). This method therefore cannot be used without hydrolysis to differentiate bound- from free-contaminating hydroxylamine.

⁴ High concentrations of protein digests caused a decrease in the yellow "blank" ferric chloride color during the Lippmann test.

at or above pH 7. The color is distinctly different from that given by the same compounds in hydrochloric acid containing 1% ferric chloride (0.6 *M*) (11). The intensity of color, and thus presumably the extent of complex fixation of the iron at neutrality is greatly dependent upon the presence of a large excess of either the hydroxamic acid or the iron. At relatively high concentrations of iron (0.7 *mM*) the latter yields some color under the slightly alkaline test conditions even in the absence of hydroxamic acids, and only slightly more color appears if the concentration of succinhydroxamic acid is 0.2–0.7 *mM*. When the latter is in excess (7 *mM*), iron in 0.02–0.2 *mM* concentrations gives color proportional to its concentration, although dependent upon the nature of the buffer.⁵

When the color intensity given by a constant low concentration of iron (0.036 *mM*) under the standard assay conditions in the presence of varying amounts of succinhydroxamic acid or hydroxylamido proteins was compared, a maximum Klett reading of about 50 was obtained with 14 *mM* succinhydroxamic acid; with amounts up to 6 mg. protein/ml. (*i.e.*, 2.5 *mM* in regard to $-\text{CO}-\text{NHOH}$ groups) readings of 60–80 were obtained, without a maximum having been obtained. Thus it appears that the protein-bound hydroxamic acids are somewhat more efficient iron-binders than succinhydroxamic acid. In contrast, however, only 2 mg. conalbumin/ml. (0.04 *mM* in reactive groups) is needed to obtain a reading of 50, which is maximal since all the iron in the solution is bound. Another means of comparing the affinity for iron of hydroxylamidoproteins and conalbumin was possible by a modified equilibrium dialysis technique (3). When buffered solutions, containing 20–35 mg. of hydroxylamido-ovomucoid and 28 $\mu\text{g.}$ iron in 7 ml. were dialysed against similarly buffered iron-free conalbumin (30 mg.), no measurable iron-complex color appeared in the latter solution. In terms of binding sites, however, this means that about 10 $\mu\text{equiv.}$ of bound hydroxamic acid immobilized 0.5 $\mu\text{equiv.}$ of iron. However, when the solution in the bag contained only 3 mg. hydroxylamidoprotein (1.2 $\mu\text{equiv.}$), an appreciable part of the iron appeared in the conalbumin solution (0.6 $\mu\text{equiv.}$ of binding sites).

The absorption spectrum of the iron complexes of hydroxylamidoproteins and succinhydroxamic acid (pH 7.6) are similar, showing a

⁵ Citrate depresses or prevents color formation; phosphate and/or bicarbonate favor it.

gradual rise from 400 to 300 $m\mu$ without a definite maximum in the visible, as is characteristic for conalbumin and siderophilin (at 460 $m\mu$) (2,12).

Effects of Denaturation and Chemical Modification

Conalbumin has been found to be inactivated by a variety of denaturing agents. The activity was also found to be dependent upon the integrity of most of the amino, as well as many other types of protein groups (carboxyl, amide, guanidyl, phenol, *etc.*) (3).

In contrast, denaturation of hydroxylamido proteins by heat, detergents, urea, or guanidine salts did not decrease their ability to give colored iron complexes in acid, or above neutrality. Neither esterification of the carboxyl groups (7) nor succinylation of the amino groups (3) affected the iron-binding activity of the hydroxylamidoproteins, in contrast to that of conalbumin. However, acetylation with acetic anhydride at pH 8-6 was found to lead to inactivation of both types of iron-binding proteins. When hydroxylamine or hydroxamic acids were treated in a similar manner with an excess of acetic anhydride, these compounds also lost all iron-binding power. It soon became evident that this was due to acetylation of the hydroxyl group.⁶ In contrast, succinic anhydride even in great excess did not cause acylation of the hydroxyl group in aqueous solution at pH 6. The latter thus appears to be a more specific reagent for selective acylation of amino groups, at least if *N*-hydroxyl groups are also present.

Effect of Enzymatic Digestion on Conalbumin and Hydroxylamidoproteins

Treatment of conalbumin with trypsin or crude pancreatin, unless preceded by heat denaturation, caused digestion and inactivation of only part of the protein, even after several days. An appreciable fraction remained nondialyzable and apparently unaffected. If the iron complex was first formed, it retained its color for a long time in the presence of trypsin at 35°. In contrast, pepsin caused partial digestion and complete inactivation of native conalbumin, rendering four-fifths

⁶ Acetic anhydride in aqueous solutions of pH 5-7 does not cause measurable acetylation of phenolic or aliphatic hydroxyl groups of proteins (8,13), but apparently only of —N—OH groups.

TABLE II
*Effect of Enzymatic Digestion on Iron-Binding Activity of
 Conalbumin and Hydroxylamido Proteins*

| Protein | Enzyme treatment | $\text{NH}_2\text{-N}^{\circ}/\text{N}$ ratio | Chromo- genic activity |
|---------------------------------------|--------------------------------------|--|------------------------------|
| Conalbumin | Pepsin (24 hr.), 0.01 N HCl | 0.14 ^b | 0% |
| Conalbumin | Pepsin, then pancreatin (48 hr.) | 0.28 | 0% |
| Conalbumin | 0.01 N HCl | 0.06 | 81% |
| Conalbumin | Pancreatin (48 hr.) | 0.18 ^c | 68% ^c |
| | Pancreatin (72 hr.) | 0.30 | 38% |
| Conalbumin, heat-denatured | Pancreatin (48 hr.) | 0.31 ^b | 0% |
| Hydroxylamido BSA | Pepsin (24 hr.) | 0.10 | +++ ^d |
| | Pepsin, then pancreatin (24 hrs.) | 0.24 | ++ |
| | Pancreatin (72 hr.) | 0.32 | + |
| Hydroxylamido BSA, heat- denatured | Pancreatin (24 hr.) | 0.23 ^e | ++ |
| | Pancreatin (24 hr.) | 0.25 ^e | ++ |
| Hydroxylamidoovomucoid | Pepsin (24 hr.) | 0.16 ^f | ++ |
| | Pepsin, then pancreatin (24 hr.) | 0.22 | ++ |
| | Pancreatin (48 hr.) | 0.10 ^f | ++ |
| Hydroxylamidoovomucoid, htd. | Pancreatin (72 hr.) | 0.21 | ++ |

^a Three-minute reaction periods were used, and the manometric van Slyke apparatus.

^b Eighty to 90% of the protein had become dialyzable.

^c When such digests were thoroughly dialyzed, about half of the original protein (of unchanged amino nitrogen), and all of the activity were retained in the bag. Thus it appears that part of the conalbumin is digested and inactivated, while the rest of the protein remains intact.

^d Since the color reaches no maximum within a practical range of excess iron or protein (see text) no quantitative evaluation is possible. Comparative chromogenic values, obtained under identical conditions, are indicated by + signs; +++ is that of the undigested hydroxylamido protein. Analyses for iron-binding in acid solution (11) indicate retention of 85-95% of the original —CO—NHOH groups in all digests. Higher values (by 7-18%) are obtained when the digests (or control solutions of the same pH) are first treated with succinic anhydride at pH 6, indicating that the slight loss in hydroxamic acid groups is due to hydrolysis.

^e The corresponding digestion figures for native and denatured BSA are 0.13 and 0.23.

^f For undigested samples this ratio is 0.05. After pepsin digestion (24 hr.) 32% of the protein remained nondialyzable, after 48 hr. digestion with pancreatin 69%.

of it dialyzable within 1 day. The acid alone (0.01 *N*) caused only a slight loss of activity, but incipient denaturation by the acid medium might nevertheless explain the susceptibility of conalbumin to inactivation by pepsin.

The hydroxylamidoproteins were more susceptible to pancreatin than to pepsin, but no more readily digested after heat denaturation. Enzymatic degradation had no effect on the iron-binding capacity in acid. At neutrality the digested protein gave somewhat less color than the original, but more color than an equivalent amount of succinylhydroxylamine. It appears that this is due to the higher chromogenic activity of the protein-bound hydroxamic acid under the test conditions.

Model Experiments with Hydroxylamine and Bicarbonate

Fiala and Burk (1) stated that "under appropriate conditions" hydroxylamine plus bicarbonate gave a complex with ferric iron with the same absorption spectrum maximum as conalbumin. A search for appropriate conditions has now demonstrated that an approximately 50 *mM* solution with regard to both hydroxylamine and bicarbonate gives a colored complex of proportional intensity to the iron content over the range of 0.02–0.2 *mM* iron. The intensity is similar to that of 7 *mM* succinylhydroxylamine (which requires no bicarbonate) with the same amounts of iron. However, if the concentration of either hydroxylamine or bicarbonate is lowered in attempts to simulate the conalbumin system, the effective concentration of the hydroxycarbamic acid is lowered to such an extent that little if any color is given upon the addition of iron. This is remarkably different from the effect of carbonate on the conalbumin-iron complex. Schade *et al.* (2) state that carbonate is essential for full color development of siderophilin, but that this requirement is fulfilled when equimolar amounts of iron and carbonate are present. This would mean that the concentration of all participants in this reaction must be of the order of 0.03 *mM*, one-thousandth of that needed to produce the hydroxycarbamic acid (—NOH—COO—) in the model system. Actually, evidence has been presented here that carbonate is not a prerequisite for the development of the colored conalbumin-iron complex.

Another point of marked difference is revealed by the addition of other buffer ions to the two systems. While conalbumin gives maximal

color in a variety of buffer media,⁷ many of these, notably phosphate and citrate, greatly depress or abolish the iron-complexing activity of the hydroxylamine-bicarbonate system.

DISCUSSION

Some of the main points of difference in the behavior of conalbumin on the one hand, and the hydroxylamidoproteins and succinylhydroxamic acid on the other hand, are listed in Table III. It is evident that

TABLE III

Comparison of Iron-Binding Characteristics of Conalbumin and Hydroxamic Acids

| Treatment | Conalbumin | Hydroxylamido-protein | Succinyl-hydroxylamine |
|--|---------------------------------------|------------------------|------------------------|
| Heat denaturation | Inactivates | No effect | — |
| Sodium dodecylsulfate denaturation | Inactivates | No effect | — |
| Pepsin digestion | Inactivates | No effect ^a | — |
| Acetic anhydride | Inactivates | Decreases activ. | Inactivates |
| Succinic anhydride | Inactivates | No effect | No effect |
| Esterification | Inactivates | No effect | — |
| Iron complex in 0.5 <i>N</i> HCl | No ^b | Yes | Yes |
| Iron complex at pH 7.6 | | | |
| Color | Salmon pink (peak at 460 m μ) | Yellow (no peak) | Yellow (no peak) |
| Hydrosulfite | No rapid effect | Decolorizes | Decolorizes |
| Excess of either reagent needed for complete reaction of the other | 0.1 equiv. | 70 equiv. | 400 equiv. |

^a Decreased activity when assayed at pH 7.6, but not in acid solution.

^b Not even after treatment with succinic anhydride which should transform any —NH—OH into the reactive hydroxamic acid group.

agents which attack only secondary valence forces (*i.e.*, denaturing agents) inactivate conalbumin but not the artificial iron-binding proteins. Similarly, an attack on the peptide chains by enzymatic degradation inactivates only conalbumin. Chemical agents which modify selectively the amino or carboxyl groups also inactivate conalbumin but not the hydroxylamidoproteins.

⁷ However, phosphate was shown to raise the pH needed for stoichiometric complex formation by one unit (2,12).

A crucial point of difference between the natural and the artificial iron-binding proteins is, obviously, the absence of —NHOH groups from the former. Alkylhydroxylamines give the same typical color test (with ferric chloride in acid) after treatment with succinic anhydride which acylhydroxylamines give directly, even if the acyl group pertains to a protein; yet no positive test for hydroxylamino compounds has been obtained with amounts up to 50 mg. of conalbumin, either in the native form, or after enzymatic or acid degradation. These analyses indicate that no more than about 0.5 R—NHOH groups can be present per mole, in contrast to the two atoms of iron bound by conalbumin. Bound hydroxylamine (—CO—NHOH) was found after hydrolytic liberation in trace amounts (0.06–0.26 equiv./mole) in various proteins, including conalbumin, egg albumin, and 60% pure siderophilin; but the small amount found and its occurrence in inactive proteins deprive it of any significance in relation to the problem under study.

Further differences are in the color and absorption spectrum of conalbumin, as compared to the hydroxylamido compounds, and in the stability of iron fixation. The great difference in dissociation constants is apparent from the fact that equivalent amounts of conalbumin and iron give over 90% of the maximal color, even at concentrations as low as $3 \times 10^{-5} M$. In contrast, $10^{-2} M$ succinhydroxamic acid or $10^{-3} M$ protein-bound —CO—NHOH groups are needed for near maximal color development at low iron concentration ($3.6 \times 10^{-5} M$). Even more marked is the difference between conalbumin and Fiala's model system, hydroxylamine and bicarbonate. In the latter case full color development is dependent upon high concentrations of both hydroxylamine and bicarbonate and is greatly depressed or prevented by the presence of other ions which favor color development in the case of conalbumin.

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SUMMARY

1. Hydroxylamidoproteins give colored complexes with iron under conditions similar to those required by the natural iron-binding proteins, conalbumin and siderophilin. The activity of the hydroxylamido-

proteins, in contrast to that of conalbumin, is retained after denaturation, enzymatic digestion, and chemical modification of amino and carboxyl groups. Only attack on the hydroxylamino group abolishes the iron-binding activity.

2. A thorough search has revealed no evidence for the occurrence of hydroxylamino or hydroxylamido groups of any kind in conalbumin.

3. Marked differences are demonstrated in the stability, color, and other qualities of the iron complexes of conalbumin and siderophilin on the one hand, and those of hydroxylamidoproteins, simple hydroxamic acids, and *N*-hydroxycarbamic acid on the other.

4. It is thus concluded that the iron-binding activity of conalbumin is not mediated by a hydroxylamine group.

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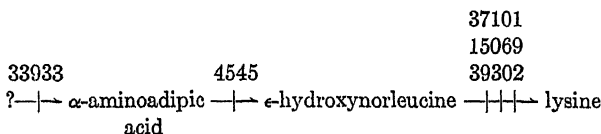
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LETTERS TO THE EDITORS

ϵ -Hydroxynorleucine as a Substitute for Lysine for *Neurospora*

Doermann (1) provided evidence for the existence of four and probably five genetically different types of lysine requiring mutants of *Neurospora*. Subsequently, it was shown by Mitchell and Houlahan (2) that one of the mutants (33933) would utilize L- α -amino adipic acid as well as L-lysine and it was, therefore, suggested that the former substance is a biological precursor of the latter in the mold. Although α -amino adipic acid is produced from lysine by animal tissues (3) it is not utilized in the place of lysine by mammals (4). On the contrary, it exerts an inhibitory effect on growth and hemoglobin production of rats (5). A similar inhibitory effect was shown by Pagé (6) to result from feeding rats deaminated casein and the active anemia-producing substance was shown to be ϵ -hydroxynorleucine (hexahomoserine). The latter substance was therefore prepared and tested as a lysine substitute for the *Neurospora* mutants. Two of the mutants of different genetic constitution were found to utilize the substance for growth, as indicated in Table I.

Other types of mutants (37101, 15069, and 39302) utilize lysine but like rats (5) are inhibited by ϵ -hydroxynorleucine. Thus the following sequence of substances and mutations is proposed:



As in other series of this kind the substances indicated may be readily converted to intermediates but may not be intermediates themselves. Although there is no evidence that this is true for α -amino adipic acid, different reisolated strains of 4545 vary considerably in their quantitative growth responses to ϵ -hydroxynorleucine.

Crude preparation of the α -oxo acids corresponding to α -amino adipic acid, ϵ -hydroxynorleucine and lysine were tested and found to lack growth-supporting activity for all of the genetic types of lysine mutants. The substances were obtained by action of L-amino acid oxidase [from *Neurospora* (7)] on the appropriate amino acids.¹ These results confirm and extend the earlier observation with synthetic α -oxo adipic acid (2), indicating that the α -oxo acid analogs are not intermediates even though the D-isomers of α -amino adipic and ϵ -hydroxynorleucine are evidently utilized in the presence of the L-isomers.

¹ We are indebted to Mr. Philip Thayer for preparation of the original reaction mixtures.

TABLE I

*Growth of Neurospora Mutants in the Presence of Lysine and ϵ -Hydroxyornithine
(88 hr. growth)*

| Mutant | Mmoles compound/20 ml | Dry weight of mycelium | |
|--------|--------------------------|----------------------------------|-----------|
| | | Dl- ϵ -Hydroxyornithine | L-lysine |
| 33933 | 0.001 | mg 1.0 | mg 6.0 |
| | 0.002 | 2.4 | 12.0 |
| | 0.005 | 8.0 | 25.5 |
| | 0.01 | 28.0 | 31.6 |
| | 0.02 | 47.0 | 45.2 |
| | 0.03 | 52.4 | 54.8 |
| | | | |
| 4545 | 0.001 | 1.4 | 5.0 |
| | 0.002 | 2.8 | 9.6 |
| | 0.005 | 7.4 | 21.4 |
| | 0.01 | 12.5 | 37.4 |
| | 0.02 | 31.8 | 50.2 |
| | 0.03 | 40.1 | 56.5 |
| | | | |

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**The Competitive Inhibition of Uricase Activity by 8-Azaguanine
and by 8-Azaxanthine¹**

8-Azaguanine (5-amino-7-hydroxy-1 *H*-*v*-triazolo[*d*]pyrimidine) was found by Roblin *et al.* (1) and Kidder *et al.* (2,3) to inhibit the growth of certain cells. 8-Azaguanine is deaminated to 8-azaxanthine (5,7-dihydroxy-1 *H*-*v*-triazolo[*d*]pyrimidine) by the

¹ This research was supported by a grant from the Nutrition Foundation, Inc.

enzyme guanase (4). The present paper concerns the competitive inhibition of the enzymatic oxidation of uric acid by the above triazolo derivatives of pyrimidine.

Uricase was prepared from rat livers according to the method of Holmberg (5) with modifications that were found to be necessary.

Uricase activity was measured by the change in optical density of a uric acid solution, at a wavelength of 290 m μ (6). The reaction was followed in a 0.1 *M* pyrophosphate buffer of pH 9.0 at 25°, with and without added inhibitors. Figure 1 shows

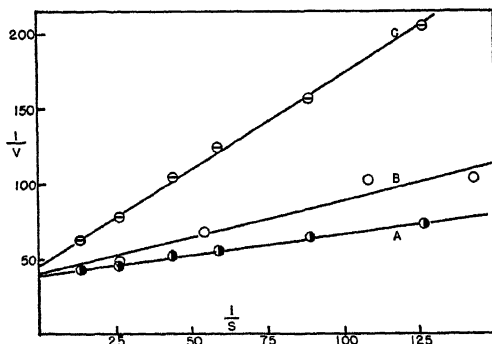


Fig. 1. Inhibition of uricase activity: curve A, reciprocal reaction rate for various concentrations of uric acid without inhibitor; curve B, reciprocal reaction rate with 8.22×10^{-5} *M* 8-azaguanine; curve C, reciprocal reaction rate with 1.49×10^{-6} *M* 8-azaxanthine.

the type of curves obtained illustrating the competitive inhibitory action of 8-azaguanine and 8-azaxanthine. The equilibrium constant (K_s) for uric acid with uricase under these conditions was 7×10^{-6} . The inhibitory constant (K_i) for 8-azaxanthine calculated according to the method of Lineweaver and Burk (7) was 5×10^{-7} , and the K_i for 8-azaguanine was 8×10^{-6} .

Uricase has no action on either 8-azaguanine or 8-azaxanthine.

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The Action of Invertase Preparations

Extracts of Jerusalem artichoke tubers produce "spot 2" (probably a trisaccharide) from mixtures of sucrose and inulin, but not in appreciable amounts from either substance alone (1). While studying this phenomenon we found that some preparations, concentrated with respect to the system hydrolyzing the tuber carbohydrates (2), produced a small amount of "spot 2" when acting on sucrose alone; this suggested that such substances might be intermediate products in the action of enzymes hydrolyzing glycosidic linkages. We, therefore, investigated the possibility that glucose and fructose might not be the only products of the action of invertase on sucrose. One milliliter of British Drug Houses "Invertase Concentrate" (prepared from yeast) was allowed to act in a total of 10 ml. of 45.5% sucrose at 17°C. Samples were withdrawn 0, 5, 10, 15, and 25 min. after the start of the reaction, boiled to inactivate the enzyme, and 5- μ l. quantities taken for paper-partition chromatography.

The chromatograms, developed with butanol-acetic (3) and sprayed with benzidine-trichloroacetic acid (4), showed the presence of at least three components with R_F values less than that of sucrose (approx. R_F values; component I, 0.085; component II, 0.060; component III, 0.047, referred to glucose, 0.18; sucrose, 0.11). These substances were present, together with fructose and glucose, in the earliest sample taken and persisted as long as sucrose remained in the reaction mixture. The final products were fructose and glucose only. A similar phenomenon has been observed by Blanchard and Albon.

Similar results have been obtained with initial concentrations of sucrose as low as 2%, with different concentrations of enzyme, and with pH values between 5 and 7. The effect was also seen when fresh baker's yeast, cytolized with ether, was allowed to act upon sucrose in phosphate-bicarbonate mixture under the conditions described by Neuberg and Lustig (5).

The presence of inorganic phosphate does not appear to be essential, since incubation with a dialyzed sample of British Drug Houses "Invertase Concentrate," 1 ml. of which gave no color with the reagents of Fiske and SubbaRow in a final volume of 20 ml., showed no detectable differences from incubation with the same enzyme preparation in the presence of 0.01 M phosphate. 2,4-Dinitrophenol in a concentration of $M/650$ similarly had no effect as judged qualitatively.

Estimations of ketose (presumably fructose) in the intermediate products during the breakdown of 10% sucrose (pH 5, 25°C.) showed after 10 min. about 5.5% of the total ketose in component III and about a fifth of this amount in each of components I and II. Chromatograms run in one direction with butanol-acetic, sprayed with invertase and run at right angles (4) indicated that glucose was present in all three spots.

The nature of these intermediate products is uncertain; that present in the greatest amount may be a trisaccharide (cf. raffinose, $R_F = 0.01$, or "spot 2" of the artichoke carbohydrates, $R_F = 0.07-0.08$, in butanol-acetic under comparable conditions). When the chromatograms were run with phenol it was not possible to decide how many spots of lower R_F value were present, but one spot ran faster than fructose, disappearing like the slower ones at the final stage of the reaction.

During the action of the same preparation on raffinose no extra spots, other than raffinose, melibiose, fructose, and a slight trace of glucose, could be distinguished.

The results suggest a more complex action of invertase than that at present accepted; alternatively a second enzyme acting on sucrose may be present. In view of the qualitatively similar results with cytolysed yeast, and with the partially purified invertase concentrate, the latter possibility would seem the less likely.

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